BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR DISOPROXIL FUMARATE AND LAMUVIDINE IN HUMAN PLASMA BY USING RP-HPLC

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

April-2012

DEPARTMENT OF PHARMACEUTICAL ANALYSIS
KMCH COLLEGE OF PHARMACY,
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CERTIFICATE

This is to certify that the dissertation work entitled**" BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR DISOPROXIL FUMARATE AND LAMUVIDINE IN HUMAN PLASMA BY USING RP-HPLC "** Submitted by Mr. **Srikanth Reddy Tiyyagura** a bonafide work carried out under the supervision and guidance of **Prof. K.Suresh Kumar, M.Pharm., (Ph.d.,)** to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy in Pharmaceutical Analysis** at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, during the academic year **2011-2012**.

I wish for his best career,

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I wish for his best career,

Prof. **K. Suresh Kumar, M.Pharm., (Ph.D.,)**

DECLARATION

I do here by declare that the dissertation work entitled " **BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR DISOPROXIL FUMARATE AND LAMUVIDINE IN HUMAN PLASMA BY USING RP-HPLC"** Submitted to The **TamilNadu** Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmaceutical Analysis**,** was done by me under the guidance of **Prof**. **K.Suresh Kumar, M.Pharm.,(Ph.d)., t**he Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, during the academic year **2011-2012**.

I abide that all the data presented in this report will be treated with utmost confidentiality.

Srikanth Reddy Tiyyagura

(Reg no: 26107227)

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **" BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR DISOPROXIL FUMARATE AND LAMUVIDINE IN HUMAN PLASMA BY USING RP-HPLC "** Submitted by Mr. **Srikanth Reddy Tiyyagura** University **Reg. No: 26107227** to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmaceutical Analysis is a bonafide work carried out by the candidate at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore and was evaluated by us during the academic year 2011-2012.

Examination Center: KMCH College of Pharmacy, Coimbatore.

Date

Internal Examiner **External Examiner**

Convener of Examination

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Srikanth reddy Tiyyagura

ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
UV	Ultra violet
BA	Bioavailability
M. W.	Molecular weight
e.g.	Example
i.e.	That is
%	Percentage
PDA	Photo Diode Array
ACN	Acetonitrile
CDSCO	Central Drug Standard Control Organization
RF	Response Factor
Mg	Milligram
Ml	Milliliter
µg	Microgram
W/w	Weight by weight
V/v	Volume by volume
µg/ml	Microgram per milliliter
ng /ml	Nanogram per milliliter
pH	Hydrogen ion concentration
^{0}C	Degree centigrade

T	Time
Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
Tab.	Table
M.P.	Melting Point
CV	Coefficient of variance
RSD	Relative standard deviation
Rpm	Rotation per min

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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR DISOPROXIL FUMARATE AND LAMUVIDINE IN HUMAN PLASMA BY USING RP-HPLC

Abstract: A simple reverse-phase high-performance liquid chromatographic method for the estimation of tenofovir disoproxil fumarate and lamivudine in human plasma samples has been developed and validated. The assay of the drugs was performed on a Phenomenex C_{18} Column with UV detection at 259 nm. The mobile phase consisted of 0.05% Heptane sulphonic acid: acetonitrile in the ratio of 80:20, and a flow rate of 1 ml/min was maintained. Linearity in the range of 200-1000 ng/ml for Tenofovir (r^2=0.998) and that of Lamivudine was found to be 200 to 1000ng/ml (r^2=0.998). Analytic parameters have been evaluated. Intra-day and inter-day precision as expressed by relative standard deviation was found to be less than 2%. The method has been applied successfully for the estimation of tenofovir disoproxil fumarate and lamivudine in spiked human plasma samples.

Keywords: Lamivudine, Tenofovir disoproxil fumerate, high-performance liquid chromatographic

I. INTRODUCTION [1-11]

The assay methods must be able to withstand the scrutiny of national drug registration authorities who judge them on the basis of criteria established by international consensus.

In bioanalytical chemistry the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma and urine) or tissue was carried out .It play a significant role for the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data[1]. The main analytical phases that comprises bioanalytical services are,

- Method development
- Method validation
- Sample analysis

Owing to increased independence among countries in recent times it has become necessary for results of many analytical methods to be accepted internationally. Consequently, to assure a common level of quality, the need for and use of validated methods has increased[2].

A full validation requires a high workload and should therefore only start when promising results are obtained from explorative validation performed during the method development phase[3]. The process of being validate a method cannot be separated from the actual development conditions, because the developer will not known whether the method conditions were being acceptable until validation studies are performed[4] . Method development clears the way for the further process on the validation stage. However, this effort is repaid by the time saved when running method routinely during sample analysis.

1.0. METHOD DEVELOPMENT.

A bioanalytical method is a set of all of the procedure involved in the collection, processing, storing, and analysis of a biological matrix for an analyte[5]. Analytical methods employed for quantitative determination of drug and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics[6].

Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. To start these work an

extensive literature survey, reading work done on the same or similar analyte and summarizing main starting points for future work is of primary importance. Based on the information from the survey, the following can be done.

- Choice of instrument that is suitable for the analysis of analyte of interest.
- Choice of the column associated with instrument of choice, the detector and the mobile phase.
- Choice of internal standard, (It must have similar chromatographic properties of analyte).
- Choice of extraction procedure (which is time economical, gives the highest possible recovery without interferences and has acceptable accuracy and precision).

Another important issue in method development stage is the choice of internal versus external standardization is common in bioanalytical methods especially with chromatographic procedures .For internal standardization, a structural or isotopic analogue of the analyte is added to the sample prior to sample pre-treatment and the ratio of the response of analyte to that of the internal standard is plotted against the concentration[7]. Another important point is that the tests performed at the stage of method development be done with same equipment that will actually be used for subsequent routine analysis.

1.1. BIOPHARMACEUTICAL ANALYSIS

1.1.1. Need for biopharmaceutical analysis

Methods of measuring drugs in biological media are increasingly important related to following;

- Bioavailability and bioequivalence studies
- New drug development
- Clinical pharmacokinetics
- Research in Basic Biomedical and Pharmaceutical Sciences.

1.2 ASSAY OF DRUGS AND THEIR METABOLITES

A number of allusions have been made to analytical methods that distinguish drugs from their metabolites. Drug metabolism can be divided into phase I and phase II transformation condensation of drugs or their phase I metabolites with common body constituents (sulfate, glucuronic acid).

This involves glucoronidation, sulfation, amino acid conjugation, acetylation and methylation. Except for reduction processes, most phase I and phase II reactions yield metabolites that are more polar and hence more water soluble than parent drug. Assay must distinguish between drug and its metabolites. If this fact is ignored, erroneous data may be generated.

1.3. ANALYSIS OF DRUG IN VARIOUS BIOLOGICAL MEDIA

The most common samples obtained for biopharmaceutical analysis are blood, urine, and feces, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized includes saliva, breath and tissue.

The choice of sampling media is determined largely by the nature of used in the study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and saliva. A bioavailability study may require drug level data in blood and / or urine whereas a drug identification or drug abuse problem may be solved with only one type of biological sample.

Detection of drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involved i.e. solvent extraction and chromatography are employed to effectively separate drug components form endogenous biological materials. The sensitivity and selectivity of the assay method was limited by the efficiency of the clean up methodology.

If the blood is allowed to clot and is then centrifuged, about 30 to 50% of the original volume is collected as serum (upper level). Thus, plasma generally is preferred because of its greater yield from blood. Blood, serum or plasma samples can be utilized for bioanalytical studies and may require protein denaturation steps before further processes.

If plasma or serum is used for the analytical procedure, the fresh whole blood should be centrifuged immediately at 5000 rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as pasture pipette, to a clean container of appropriate size of storage.

Urine is the easiest one to obtain from the patient and also permits collection of large and frequently more concentrated samples. The lack of protein in a healthy individual's urine obviates the need for denaturation steps, because urine samples are readily obtained and often provide the greatest source of metabolites, they are frequently analyzed in drug metabolism studies.

1.4 STORAGE REQUIREMENTS FOR BIOLOGICAL SAMPLES

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride was immediately added after collection helps to prevent decomposition.

When collecting and storing biological samples, the analyte should be contaminated with storage vessels. For example, plastic –ware frequently contains high boiling liquid bis (2-ethylhexyl) phthalate; similarly, the plunger-plugs of vacutainers are known to contain tri-butoxyethyl phosphate, which can be interfere with certain drug analysis.

1.5 BIOANALYTICAL METHODOLOGY

The bioanalytical methods used to determine the drug and/or its metabolites in the plasma, serum, blood or urine or any other suitable matrix must be well characterized, standardized, fully validated and documented to yield suitable results that can be satisfactorily interpreted.

Although there are various steps in the development and validation of an analytical procedure, the validation of the analytical method can be envisaged to be to consist of two distinct phases:

a) The ***pre-study phase*** which comes before the actual start of the study and involves the validation of the method on biological matrix human plasma samples and spiked plasma samples.

b) The ***study phase*** in which the validated bioanalytical method is applied to the actual analysis of the samples from bioavailability and bioequivalence studies mainly to confirm the stability, accuracy and precision[9].

1.5.1. Pre-studies Phase

The following features of the bioanalytical method must be evaluated and documented to ensure the acceptability of the performance and reliability of analytical results.

- ***Stability of the drug metabolite in the matrix:***

Stability of the drug and/or its active metabolite in the biological matrix under the conditions of the experiment (including any period for which samples are stored before analysis) should be established. The stability data should also include the influence of at least three freezing and thawing cycle's representatives of actual sample handling. The absence of any sorption by the sampling containers and stoppers should also be established.

- ***Specificity/ selectivity:***

Data should be generate to demonstrated that the assay does not suffer from the interference by the endogenous compounds, degradation products, other drugs likely to be present in study samples, and metabolites of drug(s) under study.

- ***Sensitivity:***

Sensitivity is the capacity of the test procedure to record the small variations in concentration. The analytical method chosen should be capable of assaying the drug/metabolites over the expected concentration range. A reliable lowest limit of quantification should be established based on an intra-and inter- day coefficient of variation usually not greater than 20 percent. The limit of detection (the lowest concentration that can be differentiated from background levels) is usually lower than the limit of quantification and the limit of detection should be identified as, "Below Quantification Limits."

- ***Precision and Accuracy:***

Precision (the degree of reproducibility of individual assays) should be established by replicate assays on standards, preferably at several concentrations. Accuracy is the degree to which the 'true' value of concentration of drug is estimated by the assays. Precision and accuracy should normally be documented at three concentrations (low, medium, high) where 'low' is in the vicinity of the lowest concentration to be measured , 'high' is a value in the vicinity of the C_{max} and the 'medium' is a suitable intermediate value.

Intra-assay precision (within days) in terms of coefficients of variation should be no more than 15%, although no more than 20% may be more realistic at values near the lower limit of quantification. Inter-assay precision (between days) may be higher than 15% but not more than 20%.

Accuracy can be accessed in conjunction and precision and is a measure of extent to which measured concentration deviates from true or nominal concentrations of analytical standards. In general, an accuracy of ± 15% should be attended.

- ***Recovery:***

Documentation of extraction recovery at high, medium and low concentrations is essential because methods with low recovery are, in general, more prone to inconsistency. If recovery was low, alternative methods were investigated. Recovery of any internal standard used should also be accessed.

- ***Range and Linearity:***

The quantitative relationship between concentration and response should be adequately characterized over the entire range of expected sample concentrations. For linear relationships, a standard curve should be defined by at least five concentrations .if the concentration response function was found to be non-linear, additional points would be necessary for the non-linear portions of the curve. Extrapolation beyond the standard curve was not acceptable.

- ***Analytical System Stability:***

To ensure that the analytical system remains stable over the time course of the assay, the reproducibility of the standard curve should be monitored during the assay. A minimal

requirement would be to run analytical standards at the beginning and at the end of the analytical run[8].

1.5.2. Study Phase

In general, with suitable variability as defined by validation data, the analysis of biological sample can be done by single determination without a need for a duplicate or replicate analysis. The needs for duplicate analysis should be assessed on a case by case basis. A procedure should be developed that document the reasons for re-analysis.

A standard curve should be generated for each analyte and used to calculate the concentration of analyte in the unknown sample assayed with that run. It is important to use a standard curve that will cover the entire range of concentration in unknown samples. Estimation of unknown samples by extrapolation of standard curves below the lowest standard concentration or above the highest standard concentration is should be re-assayed after dilution.

1.7. EXTRACTION PROCEDURE FOR BIOLOGICAL SAMPLE

After pre treating biological material, the next step is usually the extraction of the drugs from the biological matrix. All separation procedures used one or more treatments of matrix – containing solute with some fluid. If the component are a liquid (extracting solvents) and a solid (e.g., lyophilized feces), it is an example of liquid solid extraction. If the extraction involves two liquid phases, it is an example of liquid-liquid extraction.

1.7.1. Precipitation:

This technique mainly depends on precipitation of matrix protein. The acids like trichloro acetic acid and perchloric acid or solvents like methanol and acetonitrile are used in various proportions to precipitate the proteins. This method is not advisable for LC-MS / GC-MS methods.

Procedure

a. **In case of acids:** Take 0.5 to 1.0 ml of plasma/serum/urine and add 100 to 200µl of 10 to 20 % perchloric acid or trichloroacetic acid or sometimes decreasing the volume and increasing the percentage of acid are also recommended.

b. In case of solvents: Take 0.5 to 1.0 ml of plasma and add 0.5 to 2.0ml of solvent methanol or acetonitrile.

After adding the acid or solvent vortex the vial for complete precipitation of protein then centrifuge and inject the supernatant. It is recommended to filter the sample whenever the technique is used to avoid clogging of the column.

1.7.2. Liquid solid extraction:

Liquid solid extractions occur between a solid phase and a liquid phase; either phase may initially contain the drug substance. Amongst the solids that have been used successfully in the extraction (usually via absorption) of drugs from liquid samples are XAD-2 resin, charcoal, alumina, silica gel, and aluminum silicate. Sometimes the drugs are contained in a solid phase, such as in lyophilized specimen. Liquid solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water.

Factor governing the absorption and elution of drugs from the resin column include solvent polarity; flow rate of the solvent through the column, and the degree of contact of the solvent within the resin beds.

In the absorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the resin surface while the hydrophilic portion of the solute remains in the aqueous phase. Alterations in the lipophilic/hydrophilic balance within the solute or solvent mix, and not within the resin affect adsorption of the solute.

Biological samples can be prepared for clean up by passing the sample through the resin bed where drug (metabolite) components are adsorbed and finally eluted with an appropriate solvent.

The liquid solid extraction method provides a convenient isolation procedure for blood samples, thus avoiding solvent extraction, protein precipitation, drug looses and emulsion formation. It is possible; however, that strong drug protein binding could prevent sufficient adsorption of drug to resin.

1.7.3. Dehydration method:

An aqueous biological sample is treated with sufficient quantity of anhydrous salt (sodium or magnesium sulfate) to create a "dried" mix. This mix is then extracted with a suitable organic solvent to remove the desired drug or metabolite.

1.7.4. Liquid – Liquid Extraction:

Liquid-liquid extraction is probably the most widely used technique because

- The analyst can remove a drug or metabolite from larger concentrations of endogenous material that might interfere with the final analytical determination.
- The technique is simple, rapid and has a relatively small cost factor per sample.
- Extract containing the drug can be evaporated to dryness, and the residue can be redissolved in a smaller volume of more appropriate solvent. In this manner, sample becomes more compatible with a particular analytic methodology in the measurement step such as mobile phase in HPLC determination.
- The extracted material can be redissolved in small volumes (e.g. 100 to 500 µL of solvent), thereby extending the sensitivity limit of an assay.
- It is possible to extract more than one sample concurrently.
- Near quantitative recoveries (90 % or better) of most drugs can be obtain through multiple or continuous extractions.

Partitioning (p) or distribution of a drug between two possible liquid phases can be express in terms of partition or distribution coefficient, usually called P.A. partition coefficient is constant only for a particular solute, temperature and pair of solvent used.

By knowing the P value for the extracted drug and the absolute volume of two phases to be utilized, the quantity of drug extracted after single extraction can be obtain. In multiple extractions methodology, the original biologic sample is extracted several times with fresh volume of organic solvent until as much drug as possible is obtained. Because the combine extracts now contain the total extracted drug, it is desirable to calculate the number of extraction necessary to achieve maximum extraction.

1.8. ESTIMATION OF DRUGS IN BIOLOGICAL SAMPLE BY HPLC

Most of the drugs in biological sample were analyzed by HPLC method because many advantages like rapidity, specificity, accuracy, and precision, ease of automation and elimination tedious extraction and isolation procedure. Some of the advantages are:

- Speed (analysis can be completed in 20 min or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phase)
- Reusable column (expensive columns but can be used for many Samples)
- Ideal for the substances of low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation leads itself to automation and quantization (less time and less labor)
- Precise and reproducible,
- Calculation are done by integrator itself and
- Suitable for preparative liquid chromatography on a much larger scale.

There are different models of separation in HPLC. They are normal phase mode, reverse phase mode reversed phase ion pair chromatography, ion exchange chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

In normal phase mode, the nature of stationary phase is polar and mobile phase is nonpolar .in this technique nonpolar compound travel faster and are eluted first because of lower affinity between the nonpolar compound and the stationary phase. Polar compound are retains for longer time and take more time to elute because of their higher affinity with the stationary phase. So the normal phase mode of separation is not generally used for pharmaceutical application because most of the drug molecules are polar in nature hence take longer time to elute.

Reversed phase mode was the most popular mode for analytical and preparative separation of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase was nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and mobile phase is a polar solvent. An aqueous

mobile phase uses secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention selectivity. The polar compound gets eluted first in this mode and nonpolar compound are retained for longer time. As most of the drug and pharmaceuticals are polar in nature, they are not retained for longer time and hence elute faster. The different columns used are octa decyl silane (ODS) or C_{18}, C_8, C_4 etc., (depending upon the increasing polarity of the stationary phase).

The various component of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or auto), guard column, analytical column, detector, recorder, and /or integrators. Recent models are equipped with computers and software for data acquisition and processing.

The choice of the column should be made after a careful consideration of the mode of the chromatographic technique. Three types of column are available based upon types of packing and particle size, namely, rigid solid, hard gels, and porous and peculiar layer beds, the column of smaller particles (3-10µ) are always preferred because they offer high efficiency(number of theoretical plates /meter) and speed of analysis.

The different types of detection used in HPLC method based on ultraviolet (UV), fluorescence, refractive index, mass spectrophotometric detector or a diode array detector (DAD) Chemical derivatization procedure for HPLC are performed in order to improve delectability, to improved selectivity (or specificity), to modify the chromatographic properties, and in some cases to provide favorable mass spectral fragmentation pattern for structural elucidation when a mass spectrometer is used either as an off or on line detector.

Methods for analyzing drug in biological sample can be developed, provided one who has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character, and solubility parameter. An exact recipe for HPLC however cannot be provided because method development involves considerable trial and error procedure. The most difficult problem arising is where to start, what type of column is worth trying with type of mobile phase. In general one begins with reverse phase chromatography, when the compounds that are hydrophilic in nature with many polar groups and are water soluble.

The organic phase, that concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best method is gradient reversed phase chromatography. Gradient can be started with 5-10% organic phase in the mobile phase and the organic phase concentration (acetonitrile or methanol) can be increased up to 100% within 20-30 min. Separation was optimized by changing the initial mobile phase composition and slope of gradient according to the chromatogram obtained from preliminary run. The initial mobile phase composition has been estimated on the basis of where the compounds of interest were eluted, namely, at what mobile phase composition.

Elution of drug molecules can be altered by changing the polarity of mobile phase. The elution strength of mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic sample (acidic or basic) can be separated; if they are present in undissociated form. Dissociation of ionic sample may be suppressed by proper selection of pH.

The pH of mobile phase has to be selected in such a way that the compounds are not ionized. If the retention time is too short, the decrease of mobile phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increased in 5% steps of the organic phase concentration is needed.

Whenever acidic or basic are to be separated it is strongly advisable to control mobile phase pH by adding a buffer, pH of the buffer be adjusted before adding organic phase. The buffer selected for a particular separation should be use to control pH over the range of $\approx$ pKa $\pm$ 1.0 the buffer should transit light at or below 220 nm so as to allow low UV detection.

Optimization can be started only after the reasonable chromatogram has been obtained. A reasonable chromatogram means that the entire compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change in mobile phase composition, the shifting of the peaks can be expected. From a few experimental measurements, the position of the peaks can be predicted within the range of investigated changes. An optimizing chromatogram is the one in which all the peaks were symmetrical and well separated in less run time.

The peak resolution was increased by using a more efficient column (column with high theoretical plate number, N), which can be achieve by using a column of smaller particle size, or

a longer column these factor, however will increased the analysis time. Flow rate does not influence the resolution, but it has a strong effect on the analysis time.

The parameter that are affected by the changes in chromatographic condition are,

- Resolution (R_s)
- Capacity factor($K^{'}$),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry Factor (As).

1.8.1. Quantitative Analysis by HPLC

Three methods are generally used for quantitative analysis in HPLC. They are the external standard method, the internal standard method and standard addition method.

- ***External standard method***

The external standard method involves the use of single standard or up to three standard solutions. So the peak area or the height of the sample and the standard used are compared directly or the slope of calibration curved based on standards that contain known concentration of the compound of interest.

- ***Internal standard method***

A widely used technique involves the addition of an internal standard to the analytes for compensating various analytical errors. In this approach, a known compound of a fix concentration is added to the known amount of sample to give separate peak in the chromatograms, to compensate for the losses of the compound of interest during sample pretreatment steps. Any loss of the compound of interest will be compromised by the loss of an equivalent fraction of internal standard. The accuracy of this approach obviously depends on the structural equivalence of the compounds of interest and the internal standard [8, 9].

Method Validation Requirements for Example (ICH) [10, 11]

Precision	
Assay repeatability	≤ 1% RSD
Intermediate precision (Ruggedness)	≤ 2% RSD
Accuracy	
Mean recovery per concentration	100.0% ±2.0%
Limit of detection	
Signal to-to-noise ratio	≥ 3:1
Limit of quantification	
Signal to-to-noise ratio	≥ 10:1
Linearity/Range	
Correlation coefficient	>0.99
y-Intercept	± 10%
Visual	Linear
Robustness	
System suitability	yes
Solution stability	± 2% change from time zero
Specificity	
Resolution from main peak	>2 min. (retention time)

2. REVIEW OF LITERATURE [12-29]

Jayaseelan *et al* [12] (2010) have reported the new analytical method development and validation for the simultaneous estimation of Lamuvidine and Stavudine in tablet dosage form by RP-HPLC Method by using reverse-phase C_{18} SYMMETRY column with mobile phase of methanol and water (80:20v/v) with UV detection at 266nm and flow rate is 1.5ml/min.

Kumar *et al* [13] (2010) have reported the method development and validation of RP-HPLC method for simultaneous determination of Lamivudine and Zidovudine using Altima C_{18} (150*4.6mm) pre packed column, filtered and degassed Ammonium acetate buffer: Methanol (80:20) as mobile phase and flow rate is 1.0ml/min and effluent was monitored at 270nm.

Rajesh and Pooja *et al* [14] (2010) have reported the Validated RP-HPLC method for simultaneous estimation of Emtricitabine and Tenofovir Disoproxil Fumarate in a tablet dosage form using Luna C18 column (25 cm*4.60 mm, particle size 5µm), mobile phase is acetonitrile: potassium dihydrogen phosphate buffer (pH 3.0 ± 0.05 adjusted with orthophosphoricacid); triethylamine (70:30:0.5 v/v), flow rate is 1.5ml/min with UV detection at 260nm.

Jayaseelan *et al* [15] (2010) have reported the Bio analytical method development and validation of Lamivudine by RP-HPLC method using C_{18} column (250mm*4.6mm, 5µm particle size), mobile phase is methanol: water (85:15v/v) with UV detection at 270nm and flow rate is 1.0ml/min.

Malipatil *et al* [16] (2009) have reported the determination of Tenofovir Disoproxil Fumarate by a sensitive simple isocratic RP-HPLC method using Luna C_{18} column (250mm*4.6mm, 5µm), mobile phase is 0.1%formic acid: acetonitrile (50:50), flow rate is 0.8ml/min with UV detection is 305nm.

Pawan *et al* [17] (2009) have reported the development and validation of a HPLC method for the simultaneous analysis of Abacavir sulphate and Lamivudine in combined tablet dosage form using Waters Nova-pak HR silica column 300mm length, mobile phase is acetic acid (0.2%v/v): methanol with UV detection at 254nm and flow rate is 2ml/min.

Sagar *et al* [18] (2009) have reported the stability indicating ion-pair RP-HPLC method for estimation of Tenofovir Disoproxil Fumarate in tablet dosage forms using C_{18}(250

mm*4mm,5µm), mobile phase is acetonitrile: 0:01M TBAHS (50:50%v/v), flow rate is 1.0ml/min and UV detection is 210nm.

Anandakumar *et al* [19](2009) have reported the validated RP-HPLC method for simultaneous Estimation of Emtricitabine and Tenofovir Disoproxil Fumarate in Pure and in Tablet Dosage Form using Luna C18 column (150 mm*4.6 mm i.d), mobile phase is acetonitrile: methanol: water (30:50:20v/v), UV detection at 258nm and flow rate is 0.6ml.

Patel *et al* [20] (2009) have reported the Spectrophtometric Method Development and Validation for Simultaneous Estimation of Tenofovir Disoproxil Fumarate and Emtricitabine in Bulk Drug and Tablet Dosage form by performing two methods, first method is formation and solving of simultaneous equation method using 259 nm and 286 nm and second method is absorption ratio method which uses at 286 nm and 247.6 nm

Seloi and Isadore *et al* [21] (2009) have reported the Rapid method for the quantitative determination of Efavirenz in human plasma using C_{18} column (2, 5µm, 150mm*2.0mm i.d) mobile phase is 0.1Mformic acid: acetonitrile: methanol (43:52:5) flow rate is 0.3ml/min with UV detection at 247nm and 275nm.

Eda *et al* [22] (2009) have reported the Development and validation of a reverse-phase HPLC method for analysis of Efavirenz and its related substances in the drug substances and in a capsule formulation using column Zorbax SB CN (15 cm*4.6mm i.d), mobile phase 90%water, with 0.05% trifluoroacetic acid/10%methanol, flow rate is 1.5ml/min with UV detection at 250nm.

Philippe Morin *et al* [23], (2005); have reported the method involved a quantitative recovery of these drugs from rat plasma by solid-phase extraction on Oasis HLB Waters cartridges followed by optimised HPLC separation on an Atlantis dC_{18} column with acetic acid–hydroxylamine buffer (ionic strength 5 mM, pH 7)-acetonitrile elution gradient. Quantitation was performed by HPLC/UV at 260 nm. Finally, a new buffer, obtained with acetic acid and hydroxylamine, has been tested in HPLC/ESI-MS/MS and appears to be an efficient volatile buffer in the medium 5–7 pH range. Indeed, at pH 7 and low ionic strength (5 mM), its buffer capacity is one hundred times higher to that obtained for the usual acetic acid/ammonia buffer.

Appala Raju *et al*[24],(2008); have reported a simultaneous stability indicating RP-HPLC method is developed for the estimation of Emtricitabine, Tenofovir Disoproxil fumerate and

Efavirenz in tablet dosage form. Chromatography was carried on an Inertsil ODS 3V column using gradient composition of 0.02M sodium dihydrogen orthophosphaste as mobile phase A and mixture of Methanol and water in ratio of 85:15 as mobile phase B at a flow rate of 1.5 ml/min with detection at 265 nm. The retention times of the Emtricitabine, Tenofovir disoproxil fumerate and Efavirenz was about 5.875, 8.800 and 12.020 min respectively.

Suresh *et al*[25], (2010); have reported a simple, accurate, precise and sensitive HPLC method with UV detection was developed and validated to separate and detected Lamivudine (3-TC) and Stavudine (internal standard) were extracted from human plasma using methanol protein precipitation and were chromatographed on a Phenomenex C_{18} column using 20µl injection volume and detection at 270nm.An isocratic mobile phase consisting of Methanol: Water (85:15%v/v) was used to separate these drugs. The absolute recoveries of 3-TC was greater than 90% were achieved. The described method can be readily utilized for analysis of pharmaceutical products.

Rajesh Sharma *et al*[26] (2009); have reported a simple, rapid reversed-phase high performance liquid chromatographic method had been developed and validated for estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form. The estimation was carried out on Luna C_{18} column with a mixture of acetonitrile: potassium dihydrogen phosphate buffer (pH 3.0 ± 0.05 adjusted with orthophosphoric acid): triethylamine in the ratio of 70:30:0.5(v/v) as mobile phase. UV detection was performed at 260 nm. The retention time was 1.78 and 2.27min. For emtricitabine and tenofovir disoproxil fumarate respectively and total run time was 4 min. at a flow rate of 1.5 mL min-1. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form.

Eunice Kazue Kano *et al*[27] By (HPLC) method with ultraviolet detection was developed to quantificate lamivudine (3-TC) in human plasma samples from bioequivalence studies. 3-TC and stavudine (internal standard, I.S.) were extracted from 0.5 ml of human plasma by acetonitrile protein precipitation. The method was validated over a concentration range of 0.05–3.00 µg/ml and used in a bioequivalence trial between two lamivudine formulations

Yadav M *et al*[28] ; have developed a selective, sensitive, rugged, and high throughput liquid chromatography tandem mass spectrometry method is developed for the determination of one nucleotide tenofovir (TFV) and two nucleosides emtricitabine (FTC) and lamivudine (3TC)

reverse transcriptase inhibitors in human plasma. Plasma samples were prepared by solid-phase extraction of the analytes and acyclovir (ACV) as internal standard using Waters Oasis MCX cartridges. The chromatographic separation is achieved in a run-time of 3.0 min on an ACE 5 CN column (150 mm × 4.6 mm, 5 μm) under isocratic conditions. The mobile phase consisted of 0.5% formic acid in water and acetonitrile (55:45, v/v). A linear dynamic range of 4.0-802 ng/mL, 15.0-3006 ng/mL, and 20.1-4023 ng/mL is established for TFV, FTC, and 3TC, respectively, using 0.2 mL plasma sample. It is successfully applied to a bioequivalence study of [300(TFV) + 200(FTC) + 300(3TC)] mg tablet formulation in 43 healthy human subjects under fasting conditions.

Anandakumar karunakaran *et al*[29] ; have developed a simple, rapid reverse - phase high performance liquid chromatographic method has been developed and validated for the simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in pure and in tablet dosage form. The estimation was carried out on a Phenomenex Luna C18 (150 mm x 4.6 mm i.d., particle size 5μm) column with a mixture of acetonitrile: methanol: water in the ratio of 30:50:20 (v/v) as mobile phase. UV detection was performed at 258 nm. The retention time was 3.27 and 4.15 min. for lamivudine and tenofovir disoproxil fumarate, respectively. The flow rate was 1.0 mL min^{-1}. The LOD and LOQ values were found to be 0.0099 and 0.0299 mg mL^{-1} for lamivudine and 0.0328 and 0.0994 mg mL^{-1} for tenofovir disoproxil fumarate, respectively.

3.1. AIM AND OBJECTIVE

Estimation of drugs in biological media is increasingly important nowadays, which reveals information like bioavailability, bioequivalence, and pharmacokinetics and drug research.

HPLC is the most suitable technique for the analysis of biological fluids owing to its well-developed characteristics and ruggedness. It is an extremely sensitive, precise, accurate, and rapid, separation technique.

From the extensive literature review it was found that very few studies have been reported for bio analytical method development and UV spectrophtometric method on Tenofovir and Lamivudine.

LC-MS/MS methods have also been reported, but this expensive apparatus would be not cost-effective for clinical routine determination of Tenofovir disproxil fumarate and Lamivudine.

With the increasing worldwide use of Tenofovir disproxil fumarate and Lamivudine, there is an urgent need to develop a simple, rapid, reliable and low-cost analytical method for the pharmacokinetic study and clinical routine monitoring.

Previous authors had used a mobile phase consisting

Yadav M et al developed a method on liquid chromatography tandem mass spectrometry for the determination of one nucleotide tenofovir (TFV) and two nucleosides emtricitabine (FTC) and lamivudine (3TC) reverse transcriptase inhibitors in human plasma with mobile phase consisting of 0.5% formic acid in water and acetonitrile (55:45, v/v) on CN column (150 mm × 4.6 mm, 5 μm).

Anandakumar karunakaran et al developed a method on HPLC and validated for the simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in pure and in tablet dosage form. The estimation was carried out on a Phenomenex Luna C18 (150 mm x 4.6 mm i.d., particle size 5μm) column with a mixture of acetonitrile: methanol: water in the ratio of 30:50:20 (v/v) as mobile phase.

HPLC:

- To develop a specific, sensitive, simple, reliable and widely applicable as well as low-cost HPLC-UV method in Human plasma.
- To separate the drug from Human plasma under the most common experimental conditions, without adding expensive special equipments.
- Literature review reveals that the previous authors used 0.5% formic acid in water and acetonitrile and acetonitrile: methanol: water. In this view, the present study aim is to replace the above acetonitrile combination of solvents by Heptane sulphonic acid and acetonitrile.

3.2. PLAN OF WORK

Bioanalytical method development for Lamuvidine and Tenofovir Disoproxil Fumarate:

The present work is planned into two phases:

Phase I:

Optimization of chromatographic conditions

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Nature of the stationary phase
- Estimation of Tenofovir and Lamivudine

Phase II:

Validation of the method

The developed method were proposed to be validated using the various validation parameters such as,

- Accuracy
- Precision
- Linearity
- Limit of detection (LOD) / Limit of quantitation (LOQ)
- Selectivity / Specificity
- System suitability.
- Ruggedness

3.3DRUG PROFILE

Lamivudine[30-31]

Chemical profile:

Chemical name : 2', 3'-dideoxy-3'-thiacytidine, commonly called 3TC

Molecular formula : $C_8H_{11}N_3O_3S$

Structure:

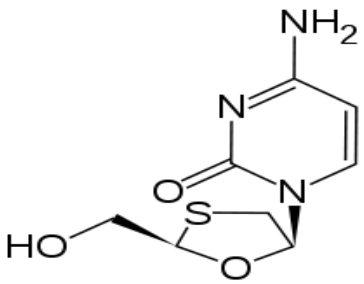


Fig 1: Structure of lamivudine

Pharmacokinetic data:

Bioavailability	- Absolute bioavailability was lies between 64 to 94%.
Protein binding	- 80 to 86%.
Half life	- 5 to 7 hours.
Routes	- Oral.
C_{max}	- 6 to 12 hours
Description	**:** White or almost white powder.
Solubility	**:** Freely soluble in water

Category:

Anti retro viral potentnucleoside is reverse transcriptase inhibitor (NRTI). Treat for chronic hepatitis B at a lower dose than for treatment of HIV.

Dose:

- For 5 mg/ day initially, titrated to response over 7-14 days, then up to a maximum dose of 10 mg once daily.
- For adults with HIV the dose is 300mg once daily or 150mg twice a day.
- For the treatment of adults with hepatitis B, the dose is 100mg once daily.
- For a child 3 months to 12 years old, about 1.4-2 mg per lb. of body weight twice a day, no more than 150 mg per dose.

Pharmacokinetics

Absorption

C_{max} is approximately 1.28 mcg/ml (single dose of 100 mg), T_{max} is 0.5 to 2 h. Absolute bioavailability is approximately 87%.

Distribution

Less than 36% protein bound. DVD is approximately 1.3 L/kg.

Metabolism

Metabolism of Lamivudine is a minor route of elimination. The metabolite is trans-sulfoxide metabolite.

Elimination

The majority is eliminated unchanged in the urine. Mean half-life is 5 to 7 h. Cl is approximately 398.5 ml/min.

Pharmacological profile

Mechanism of action:

Lamivudine is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It is phosphorylated to active metabolites that will compete for incorporation into viral DNA. They inhibit the HIV enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3'-OH group in the nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated.

TENOFOVIR DISOPROXIL FUMARATE[32-33]

Chemical profile:

Chemical name **:** ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl]oxy}methyl) phosphonic Acid.

Molecular formula **:** $C_9H_{14}N_5O_4P$ = 287.213 g/mol

Structure:

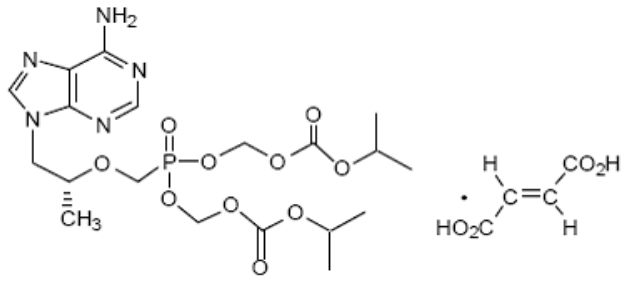


Fig 2: Structure of Tenofovir Disoproxil fumarate

Pharmacokinetic data:

Bioavailability - 25%

Protein binding - >99.5%.

Half life - 17 hours.

Routes - Oral and intravenous.

C_{max} - 0.5 to 1 hours

Description : A white powder

Solubility : Freely soluble in water, slightly soluble in methanol.

Category : Antiretroviral

Volume of distribution : Approximately 500 litter indicating additional tissue binding.

Pharmacology

Tenofovir disoproxil fumarate is a prodrug of tenofovir that inhibits the activity of HIV-1 reverse transcriptase and hepatitis B virus (HBV) polymerase by competing with deoxyadenosine 5′-triphosphate and by DNA chain termination after incorporation into DNA.

Pharmacokinetics

Absorption

Tenofovir C_{max} is approximately 0.3 mcg/mL and AUC is approximately 2.29 mcg/ml. T_{max} is approximately 1 h, and bioavailability is approximately 25%. Administration following a high-fat meal increases the oral bioavailability, with an increase in AUC of approximately 40% and an increase in C_{max} of approximately 14%.

Distribution

Vd of tenofovir is approximately 1.3 L/kg. Binding to plasma or serum proteins is less than 0.7% and 7.2%, respectively.

Metabolism

Tenofovir is not a CYP-450 substrate.

Elimination

Elimination of tenofovir is by glomerular filtration and tubular secretion. Approximately 70% to 80% is recovered in urine as unchanged drug. Elimination half-life is approximately 17 h.

Indications and Usage

Treatment of HIV-1 infection in adults and children 12 y of age and older in combination with other antiretroviral agents; treatment of chronic hepatitis B infection in adults.

Pharmacological profile

Mechanism of action:

Before phosphorylation, tenofovir disoproxil fumarate is converted to tenofovir in the intestinal lumen and plasma by diester hydrolysis. Tenofovir is then internalized into cells, possibly by endocytosis, and subsequently phosphorylated in sequential steps to tenofovir monophosphate and to the active metabolite, tenofovir diphosphate. In a mechanism similar to that of NRTIs, tenofovir diphosphate competes with its natural nucleotide counterpart, deoxyadenosine 5´-triphosphate, for incorporation into newly forming HIV DNA. Once successfully incorporated, termination of the elongating DNA chain ensues, and DNA synthesis is interrupted. Although the end results of tenofovir activity is similar to that of the NRTIs, subtle differences exist between this class of drugs and tenofovir. Like tenofovir, NRTIs also must be phosphorylated to active metabolites to inhibit reverse transcription of RNA into DNA. However, since tenofovir already contains a phosphonate group attached to the adenine base, the initial addition of a phosphate group is circumvented; thus, only two steps are required in the phosphorylation of tenofovir, as opposed to three with the NRTI class. As the initial phosphorylation step is potentially a limiting factor in the activation of NRTIs in resting $CD4^+$ cells and macrophages, tenofovir may result in better antiviral activity than NRTIs in cells that have limited proliferative and phosphorylative capacity.

3.4 METHODOLOGY

A. Materials and instrument used:

a) Drug sample & Study products:

Standard drug of Lamivudine and Tenofovir Disoproxil Fumarate were procured from Hetero Drugs Limited, Hyderabad. Andhra Pradesh, India.

Plasma: Blank plasma was collected from Kovai Medical Center and Hospitals, Coimbatore.

b) Chemicals and solvents used for estimation:

- HPLC Water (HPLC Grade) - Qualigens, Mumbai, India.
- Acetonitrile (HPLC Grade) - Rankem, Mumbai, India.
- Methanol (HPLC Grade) - Finar, Ahmadabad, India.
- Heptane sulphonic acid (HPLC Grade) - Otto Kemi Ltd, Mumbai.
- Orthophosphoric acid (AR Grade) - SD fine chem. Ltd, Mumbai.

c) Instruments used:

- Elico pH meter LI 127.
- Shimadzu LC-20 AT HPLC.
- SPD-M20A Prominence diode array detector.
- Shimadzu 1600 LC-UV Spectrophotometer.
- Sonica ultrasonic cleaner.
- Solvent filtration unit – Millipore.
- Shimadzu electronic balance AY 220.
- Ultra cooling centrifuge – Remi, India

B.OPTIMIZATION OF CHROMATOGRAPHIC CONDITION FOR THE ESTIMATION OF LAMIVUDINE AND TENOFOVIR DISOPROXIL FUMARATE

a) Selection of wavelength

An UV spectrum of 10 µg/ml Lamivudine in water and Tenofovir Disoproxil Fumarate in methanol was recorded by scanning in the range of 200 nm to 400 nm. From the UV spectrum, it was observed that Lamivudine has 272 nm and Tenofovir Disoproxil Fumarate has 259 nm as suitable wavelength.

b) Selection of chromatographic method

Selection of proper chromatographic method depends on the nature of the sample or its properties like ionic/ionizable/neutral character, its molecular weight and solubility. The drug selected for the present study is Polar in nature hence, reverse phase HPLC or ion-pair or ion–exchange chromatography method must be used. Because of its simplicity and suitability for initial separations reverse phase method was selected.

c) Initial chromatographic conditions for separation of Lamivudine and Tenofovir Disoproxil Fumarate

Standard solution:

10µg/ml of Lamivudine and Tenofovir Disoproxil Fumarate was dissolved in 10 ml of HPLC grade water.

Equipment

System	:	Shimadzu gradient HPLC
Pump	:	LC – 20AT prominence solvent Delivery system
Detector	:	SPD-M20A Prominence Diode array Detector
Injector	:	Rheodyne 7725i with 20µl loop

Chromatographic conditions 1

Stationary phase	:	Phenomenex C_{18} column.

Mobile phase	:	Solvent A: Triethylamine buffer 5mM pH 3.5 (adjusted with Orthophosphoric acid) Solvent B: Acetonitrile
Solvent ratio	:	50:50(A: B)
Wavelength of Detection	:	259 nm.
Flow rate	:	1.0ml/min.
Sample size	:	20µl.
Needle wash	:	HPLC grade water.
Temperature	:	Room temperature (25^0C)

At the above chromatographic conditions Lamivudine was eluted at the retention time of 18.2min. The peak observed was broad and asymmetric, thus not selected for further studies. Tenofovir was not eluted.

Chromatographic condition 2

Stationary phase	:	Phenomenex C_{18} Column
Mobile phase	:	Solvent A: Triethylamine 5mM pH 4 (adjusted with Orthophosphoric acid) Solvent B: Acetonitrile
Solvent ratio	:	80:20 (A: B)
Detection	:	259 nm
Flow rate	:	1.0 ml/min
Sample size	:	20µl.
Needle wash	:	HPLC grade water.

Temperature : Room temperature (25^0C)

Lamivudine was eluted at retention time of 17.1 minute with peak splitting and fronting and Tenofovir was eluted at retention time of 11.1minute with broad peak hence not selected for further method development.

Chromatographic condition 3

Stationary phase	:	Phenomenex C_{18} Column
Mobile phase	:	Solvent A: ammonium acetate buffer 20Mm pH 3.5(adjust with Orthophosphoric acid) Solvent B: Acetonitrile
Solvent ratio	:	50:50
Detection	:	259 nm
Flow rate	:	1.0 ml/min
Sample size	:	20 µl
Needle wash	:	water HPLC grade
Column temperature	:	room temperature (25ºC).

The retention time of Lamivudine was found to be 12.1min and that of Tenofovir was found to be 8.4min. The peak observed was broad and the retention time of both drugs not close to each other, so the peak separation is high observed.

Chromatographic condition 4

Stationary phase	:	Phenomenex C_{18} Column
Mobile phase	:	Solvent A: Heptane sulphonic acid 0.05% pH 2.5 (adjusted with Orthophosphoric acid) Solvent B: Acetonitrile

Solvent ratio	:	80:20(A: B)
Detection	:	259 nm
Flow rate	:	1.0 ml/min
Sample size	:	20µl.
Temperature	:	Room temperature (25^0C)

Retention time Tenofovir was found to be 4.5min and Lamivudine was found to be 9.6min. Broad peaks with tailing observed for both the drugs.

Chromatographic condition 5

Stationary phase	:	Phenomenex C_{18} Column
Mobile phase	:	Solvent A: Heptane sulphonic acid 0.05%
		PH 3.0 (adjusted with Orthophosphoric acid)
		Solvent B: Acetonitrile
Solvent ratio	:	80:20(A: B)
Wavelength of		
Detection	:	259 nm
Flow rate	:	1.0 ml/min
Sample size	:	20µl.
Temperature	:	Room temperature (25^0C)

Tenofovir was eluted at 4.8 min and Lamivudine at 8.1min with perfect peak properties, hence selected for further studies.

d) Effect of pH

Using 20% of acetonitrile and buffer solution of different pH ranging from 3.5, 4.5, 5.5 at 272 nm, the standard solution was run up to 20 min at a flow rate of 1.0 ml/min. The

retention time of Tenofovir was 5.6, 4.9, 4.1 minutes respectively. For the present study, a pH of 3.0 was selected as the chromatogram obtained with this pH was symmetrical in shape.

e) Effect of ratio of mobile phase

Heptane sulphonic acid and acetonitrile with 70:30, 75:25 and 80:20 ratios were used as the mobile phase. At 80:20 ratios, symmetric peaks were eluted at 4.2 min and 8.9 min for Tenofovir and Lamivudine respectively. At 70:30 and 75:25 ratios the peaks were asymmetrical in shape. Thus for the present study 80:20 ratio of Heptane sulphonic acid and acetonitrile was selected as the mobile phase.

f) Effect of flow rate

Keeping the mobile phase ratio at (80:20, v/v) Heptane sulphonic acid: acetonitrile, the chromatograms were recorded at a flow rate of 0.5ml/min, 1.0ml/min 1.5ml/min. At flow rate of`1.0ml/min, the peaks were sharp and separated with good resolution. Hence, 1ml/min was kept constant for the present analysis.

g) Fixed chromatographic conditions

The following chromatographic conditions were used for the estimation of Tenofovir and Lamivudine in human plasma.

Stationary phase	:	Phenomenex C_{18} Column
Mobile phase	:	Solvent A: Heptane sulphonic acid 0.05%
		PH 3.0 (adjusted with Orthophosphoric acid)
		Solvent B: Acetonitrile
Solvent ratio	:	80:20(A: B)

Detection	:	259 nm
Flow rate	:	1.0 ml/min
Sample size	:	20µl.
Temperature	:	Room temperature (25^0C)

Pretreatment method for biological fluid:

Method of sample preparation is an important criteria for biological samples. For the present study plasma was obtained from Clinical Laboratory Services of KMCH Hospital, Coimbatore. Protein precipitation method was selected for the present study.

Protein precipitation

This method is the most commonly used method for the extraction of analytes from biological fluids. In this method precipitating agents like acetonitrile, methanol mixture of acetonitrile-methanol etc was added to the blank plasma to precipitate the proteins present in the plasma and then the precipitate formed was removed by filtration or centrifugation. The supernatant resulted is directly injected into HPLC column without any further treatment and chromatograms were recorded.

An aliquot of 0.1ml of plasma in a glass tube 0.05ml of standard solution, 0.05ml of internal standard solution and 0.2 ml of mixture of acetonitrile-methanol in the ratio of (80:20,v/v) was added and then centrifuged for 15 minutes at 5000 rpm. The same extraction procedure was also repeated by using methanol and acetonitrile separately for extraction of drug from plasma and the percentage recovery was calculated for all the precipitating agents.

The chromatograms of plasma extracted with acetonitrile, methanol and mixture of acetonitrile- methanol (80:20, v/v) were recorded using the fixed chromatographic conditions. The chromatogram of blank plasma without any drug was also recorded. Based up on the percentage recovery acetonitrile-methanol mixture (80:20, v/v) was selected for the present study because of its higher percentage recovery.

Preparation of standard stock solution: Stock solution of Lamivudine and Tenofovir 1mg/ml were prepared separately by dissolving 10mg of each drug in 10ml standard flasks and the volume was made up to 10 ml with the mobile phase.

Working standards

From the stock solution working standard solutions of 100µg/ml was prepared by diluting 1ml to 10ml with mobile phase. Further solutions were made by from the above solution by diluting 0.8ml, 1.6ml, 3.2ml, 4.8ml, 6.4ml, and 8.0ml standard solutions to 10ml in a standard flask with mobile phase. An aliquot of 0.05ml from these working standards were taken in serial 10ml standard flasks to effect concentrations of 100, 200, 400, 600, 800 and 1000ng/ml respectively.

Preparation of standard graph:

Preparation of calibration standards

To 0.1ml of blank plasma 0.05ml of Lamivudine and 0.05ml of Tenofovir were added to get concentration of 100, 200, 400, 600, 800 and 1000ng/ml respectively. To these calibration standards 0.2ml of precipitating agent mixture of acetonitrile-methanol (80:20, v/v) was added and then centrifuged for 15 minutes at 5000 rpm. After centrifugation the clear supernatant liquid was collected and a quantity of 20µl was injected into the HPLC column and chromatograms were recorded. Standard calibration graph was plotted using ratio of peak area of Lamivudine and tenofovir to its concentration.

ESTIMATION OF LAMIVUDINE AND TENOFOVIR IN HUMAN PLASMA:

Recording the chromatogram

The optimized chromatographic conditions were maintained to record the chromatograms of the calibration standards Lamivudine and Tenofovir of and sample from a clinical study. First, baseline stabilization was done for about 20 minutes. Then standard solutions, calibration standard solutions and sample from clinical study containing Lamivudine and Tenofovir were injected and chromatograms were recorded.

4. RESULTS AND DISCUSSION

Bioanalytical method was developed for Tenofovir and Lamivudine and it was validated for its transferability.

Chromatographic Conditions

Acetonitrile: Methanol (80:20) was selected as good separating agent for Tenofovir and Lamivudine, since it showed maximum recovery in comparison with acetonitrile or methanol (Table1&2). The chromatogram was recorded for the standard calibration and plasma sample under developed chromatographic conditions. The retention time of Tenofovir is 4.6 minutes and that of Lamivudine is 8.1 minutes respectively. The chromatogram was well resolved without any interference from one another. Moreover, peaks not showed any tailing or fronting. The concentration of Tenofovir and Lamivudine in Human plasma was determined from the calibration of the spiked plasma by regression analysis. It showed very good linearity in the range of 200-1000 ng/ml for Tenofovir, the r^2 value was found to be 0.998 (Fig. 3) and that of Lamivudine was found to be 200 to 1000ng/ml, the r^2 value was found to be 0.998 (Fig .4).

The peaks obtained in the present study were symmetric, good and no interference was observed between the peaks.

The method developed was advantageous than the reported methods by its lesser precision values and increased accuracy values. The run time of 10 minutes makes the method rapid and economical than the previously reported methods.

The extraction method used for the present study was simple and newer than previous methods. In most of the reported methods used for plasma for extraction of drug was protein precipitated with acetonitrile but, for present method acetonitrile –methanol mixture was selected because of its maximum recovery of drug from plasma and it is advantageous.

Table: 1 Recovery study of Tenofovir

Levels	**Conc. of Drug added (ng/ml)**	**Amount of drug recovered in plasma sample (ng/ml)**		**(%) Recovery**	
		Methanol	**ACN**	**Methanol**	**CAN**
Level – I	200	192.3	195.4	96.15	97.7
Level –II	400	386.5	383.7	96.67	95.92
Level III	500	491.05	486.5	98.21	97.3

Table: 2 Recovery study of Lamivudine

Levels	**Conc. of Drug added (ng/ml)**	**Amount of drug recovered in plasma sample (ng/ml)**		**(%) Recovery**	
		Methanol	**ACN**	**Methanol**	**CAN**
Level – I	200	194.6	196.1	97.3	98.05
Level –II	400	376.3	380.3	94.07	90.15
Level III	500	484.3	481.6	96.86	96.38

Table.3 *Peak area of calibration curve for Tenofovir*

Concentration ng/ml	Peak area
0	0
200	1998
400	3997
600	5996
800	7894
1000	9993

Fig: 3 Calibration curve for Tenofovir

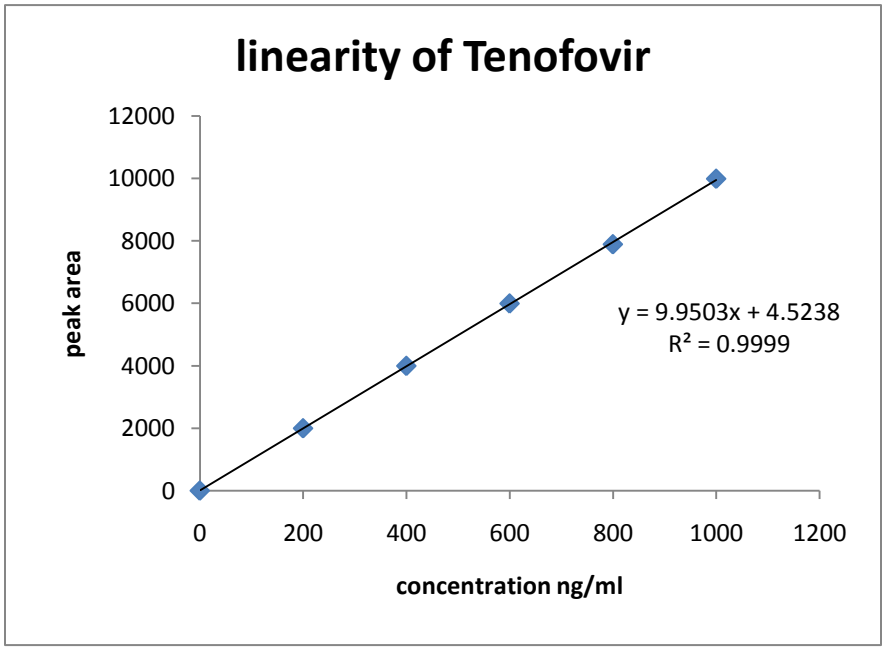
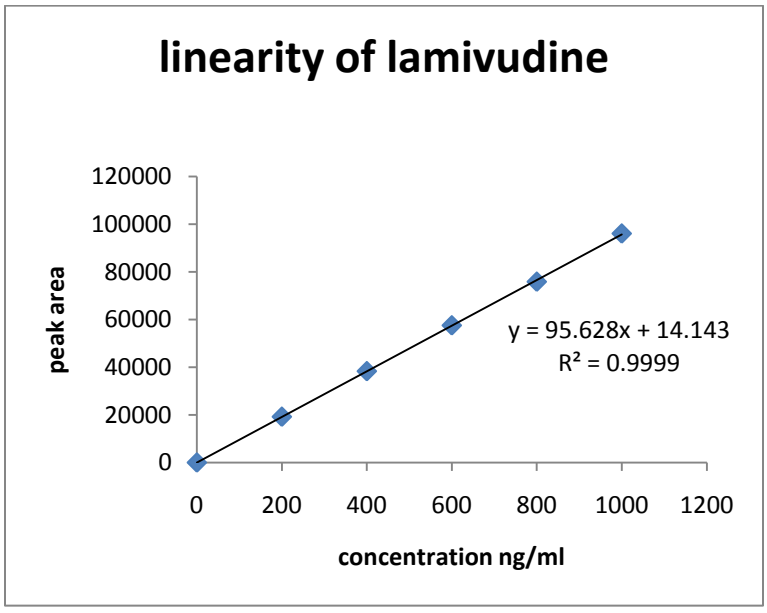


Table.4 *peak area of calibration curve for Lamivudine*

Concentration ng/ml	Peak area
0	0
200	19211
400	38322
600	57534
800	75845
1000	96056

Fig: 4 *Calibration curve for Lamivudine*



4.1 VALIDATION OF THE METHOD

Accuracy and precision

The accuracy and precision study was performed at two levels intra-day and inter-day. The developed method showed good accuracy and precision (Table5 to 8). The intra-day accuracy of Tenofovir was range between 95.02% and 100.28% with precision between 0.0192 and 0.0401, the inter-day accuracy range of Tenofovir between 97.01% and 99.78% with precision between 0.0193 and 0.0412. The intra-day accuracy of Lamivudine range was between 96.45% and 99.86% with precision between 0.0166 and 0.0389, the inter-day accuracy of Lamivudine range between 95.64% and 98.77% with precision between 0.0167 and 0.2153.

Table**:** *5 Accuracy and Precision Studies of Tenofovir (Intra - Day)*

S. No.	Conc. of Drug (ng/ml)	Mean concentration Found (ng/ml)	Accuracy (%)	Precision (%)
1	200	198.2	96.13	0.040
2	400	385.0	96.42	0.028
3	600	585.7	97.61	0.019
4	800	782.1	97.76	0.013
5	1000	975.7	97.57	0.018

*n=6 (Mean of 6 values)

Table**:** *6 Accuracy and Precision Studies of Tenofovir (Inter - Day)*

S. No.	Conc. of Drug (ng/ml)	Mean concentration Found (ng/ml)	Accuracy (%)	Precision (%)
1	200	189.1	94.55	0.044
2	400	381.8	95.45	0.019
3	600	569.5	94.91	0.031
4	800	793.1	99.13	0.018
5	1000	961.2	96.12	0.023

*n=6 (Mean of 6 values)

Table**:** *7 Accuracy and Precision Studies of Lamivudine (Intra - Day)*

S. No.	Conc. of Drug (ng/ml)	Mean concentration Found (ng/ml)	Accuracy (%)	Precision (%)
1	200	191.8	95.9	0.016
2	400	390.1	97.52	0.028
3	600	581.5	96.91	0.037
4	800	779.3	97.14	0.029
5	1000	979.4	97.94	0.031

*n=6 (Mean of 6 values)

Table**:** *8 Accuracy and Precision Studies of Lamivudine (Inter - Day)*

S. No.	Conc. of Drug (ng/ml)	Mean concentration Found (ng/ml)	Accuracy (%)	Precision (%)
1	200	183.01	91.05	0.017
2	400	376.02	94.05	0.215
3	600	575.5	95.91	0.139
4	800	786.3	98.28	0.061
5	1000	973.7	97.03	0.071

*n=6 (Mean of 6 values)

Linearity and range

According to ICH guidelines, the method proved to be linear between 200-1000 ng/ml for Tenofovir, with a correlation equation y =9.950x – 4.523, correlation coefficient was > 0.999 (fig.3). The linearity range for Lamivudine was between 200-1000 ng/ml, with a correlation equation y =95.62 x –14.14, correlation coefficient was > 0.999 (fig.4).

Limit of Detection and Limit of Quantification

The limit of quantification (LOQ) and limit of detection (LOD) of Tenofovir was found to be 311.55 ng/ml, 102.81ng/ml and Lamivudine was found to be 32.48, 10.71ng/ml respectively.

Recovery from plasma

The extraction efficiency of Tenofovir from Human plasma at the concentrations of 200, 400, and 500ng/ml was found to be 95.02%, 98.90% and 100.28%. The extraction efficiency of Lamivudine from Human plasma at the concentrations of 200, 400 and 500 ng/ml was found to be 95.7%, 96.92% and 98.3%. The results of recovery studies are shown in Table.9 and 10.

Table: 9 Recovery study for extraction method

Levels	**Conc. of Drug added (ng .ml)**	**Amount of drug recovered in plasma sample (ng /ml)**	**Relative Recovery (%)**	**%RSD**
Level – I	200	192.3	95.02	8.6
Level –II	400	386.5	98.90	7.9
Level III	500	491.05	100.28	7.3

*n=6 (Mean of 6 values)

Table: 10 Recovery studies of Lamivudine

Levels	**Conc. of Drug added (ng .ml)**	**Amount of drug recovered in plasma sample (ng /ml)**	**Relative Recovery (%)**	**%RSD**
Level – I	400	382.52	95.62	8.9
Level –II	800	771.77	96.47	9.3
Level III	1000	1583.02	98.93	8.5

*n=6 (Mean of 6 values)

System suitability

System suitability parameters such as column efficiency (theoretical plates), resolution factor and peak asymmetry factor of the optimized methods were found satisfactory. The results of stability are shown in Table 10 and 11.

Table: 11 *System suitability studies*

S. No.	Parameters	Tenofovir
1	Theoretical Plate	12438.76
2	Tailing Factor	0.958
3	HETP	12.059
4	LOD	102.81ng/ml
5	LOQ	311.55ng/ml

Table: 12 *System suitability studies*

S. No.	Parameters	Lamivudine
1	Theoretical Plate	654.76
2	Tailing Factor	0.944
3	HETP	22.93
4	LOD	10.71 ng/ml
5	LOQ	32.48ng/ml

Ruggedness

It expresses the precision within laboratories variations like different days, different analyst, and different equipments. Ruggedness of the method was assessed by spiking the standard 6 times in two different days with different analyst and the reports are shown in Table 13.

Table :13 *Ruggedness studies*

Drug	**Concentration** µgm/ml	**Mean Peak area**	**%RSD**
DAY I Analyst-1			
Lamivudine	200µg/ml	19211.1	0.46
Tenofovir	200µg/ml	1998.4	0.14
DAY 2 Analyst-2			
Lamivudine	200µg/ml	19244.2	0.42
Tenofovir	200µg/ml	1997.9	0.32

*n=6 (Mean of 6 values)

==== Shimadzu LCsolution Analysis Report ====

D:\tenofovir\Blank plasma (80 20 Heptane, acn) 129.lcd
Acquired by: K.M.C.H College of pharmacy
Sample Name: Blank plasma
Sample ID: Test
Vail #:
Injection Volume: 20 ul
Data File Name Blank plasma (80 20 Heptane, acn) 129.lcd
Method File Name: Lamivudine & Tenofovir.lcm
Batch File Name:
Report File Name: Default.lcr
Data Acquired: 8/30/2011 1:22:03 PM
Data Processed: 2/15/2012 11:32:12 AM

<Chromatogram>

D:\tenofovir\ Blank plasma (80 20 Heptane, acn) 129.lcd

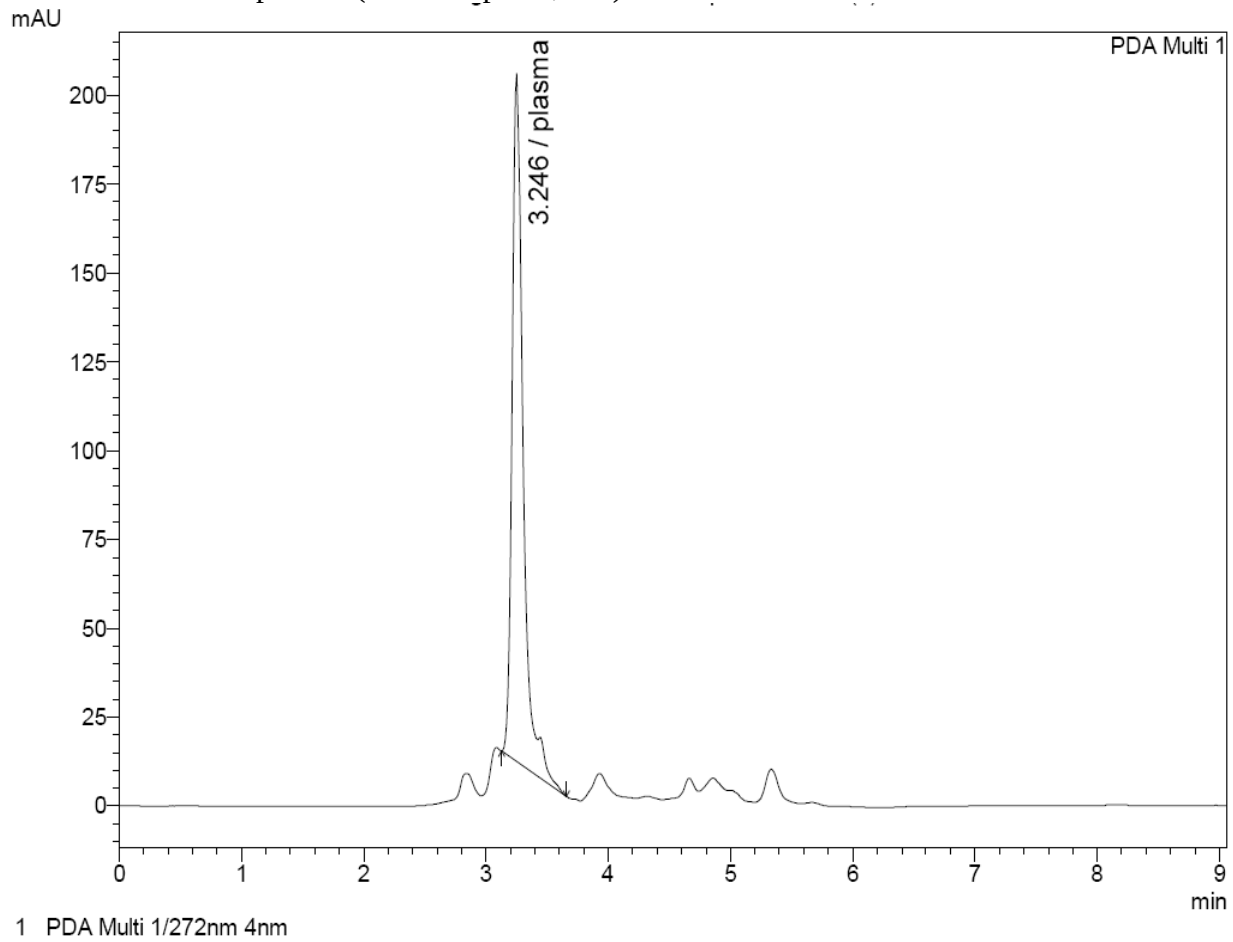


Fig: 2 Chromatogram of blank plasma

==== Shimadzu LCsolution Analysis Report ====

D:\lab solutions\STD, tenofovir & Lamivudine (80 20 hept, acn) ph 3 030.lcd
Acquired by: K.M.C.H College of pharmacy
Sample Name: combo
Sample ID: Standard
Vail #:
Injection Volume: 20 ul
Data File Name: tenofovir & Lamivudine (STD) (80 20 hept, acn) ph 3 030.lcd
Method File Name: lamivudine.lcm
Batch File Name:
Report File Name: Default.lcr
Data Acquired: 2/18/2012 1:06:59 PM
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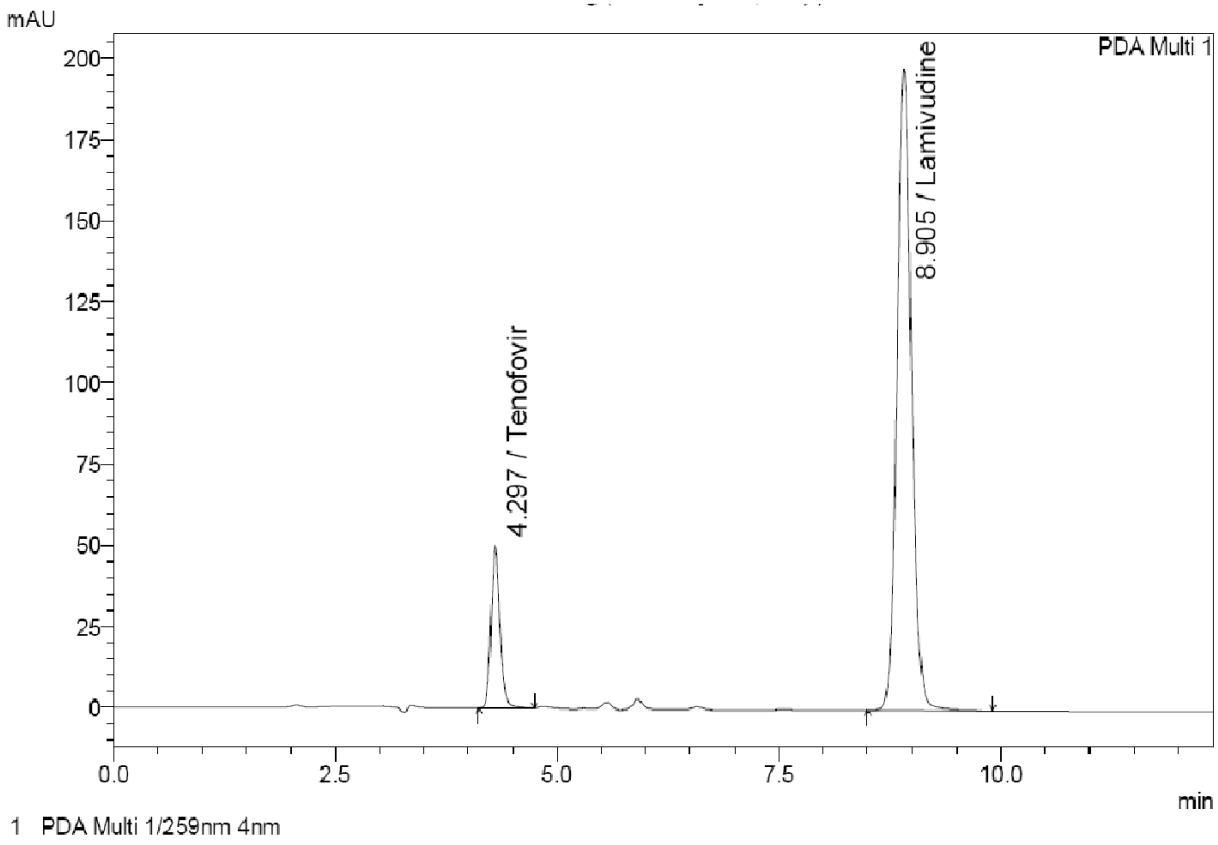

Fig: 3 Chromatogram of Tenofovir and Lamivudine 100µg/ml

==== Shimadzu LCsolution Analysis Report ====

D:\lab solutions\STD, Tenofovir & Lamivudine (80 20 hept, acn) ph 3 124.lcd
Acquired by: K.M.C.H College of pharmacy
Sample Name: lamuvidine ph 3
Sample ID: Standard
Vail #:
Injection Volume: 20 ul
Data File Name: Tenofovir & Lamivudine (STD) 10µg/ml (80 20 hept, acn) ph 3 124.lcd
Method File Name: lamivudine.lcm
Batch File Name:
Report File Name: Default.lcr
Data Acquired: 2/19/2012 2:36:59 PM
Data Processed: 2/19/2012 4:04:05 PM

<Chromatogram>

D:\lab solutions\STD, Tenofovir & Lamivudine *10µg/ml* (80 20 hept, acn) ph 3 124.lcd

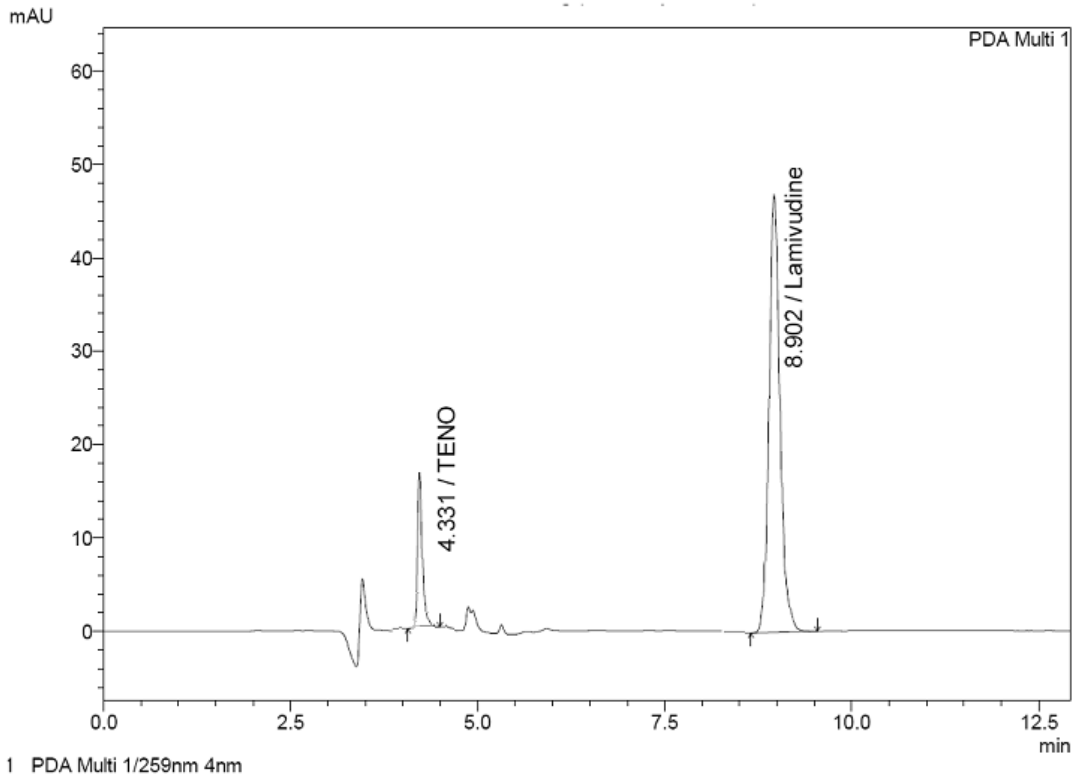

Fig: 5 Chromatogram of Tenofovir and Lamivudine 10µg/ml

==== Shimadzu LCsolution Analysis Report ====

C:\Documents and Settings\Administrator\Desktop\New Folder (2)\sample 018.lcd
Acquired by: KMCH College of pharmacy
Sample Name: plasma acn meth sample ph 3
Sample ID: Sample
Injection Volume: 20 ul
Data File Name: spiked plasma 200ng.lcd
Method File Name: fsdf.lcm
Batch File Name:
Report File Name: Default.lcr
Data Acquired: 2/21/2012 10:18:51 AM
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<Chromatogram>

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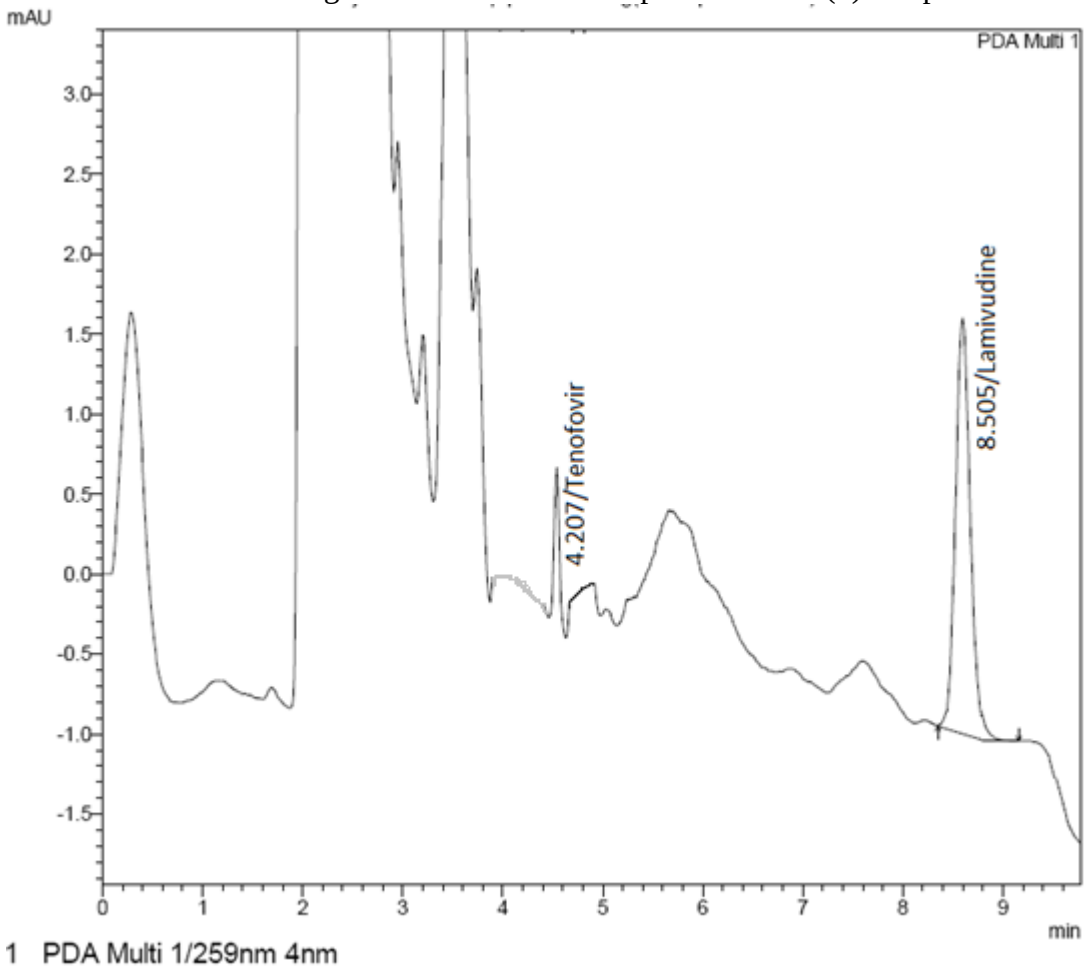

***Fig*: 10 Chromatogram of Tenofovir and Lamivudine in plasma 200ng/ml**

==== Shimadzu LCsolution Analysis Report ====

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Sample Name: plasma acn meth sample ph 3
Sample ID: Sample
Injection Volume: 20 ul
Data File Name: spiked plasma 400ng.lcd
Method File Name: fsdf.lcm
Batch File Name:
Report File Name: Default.lcr
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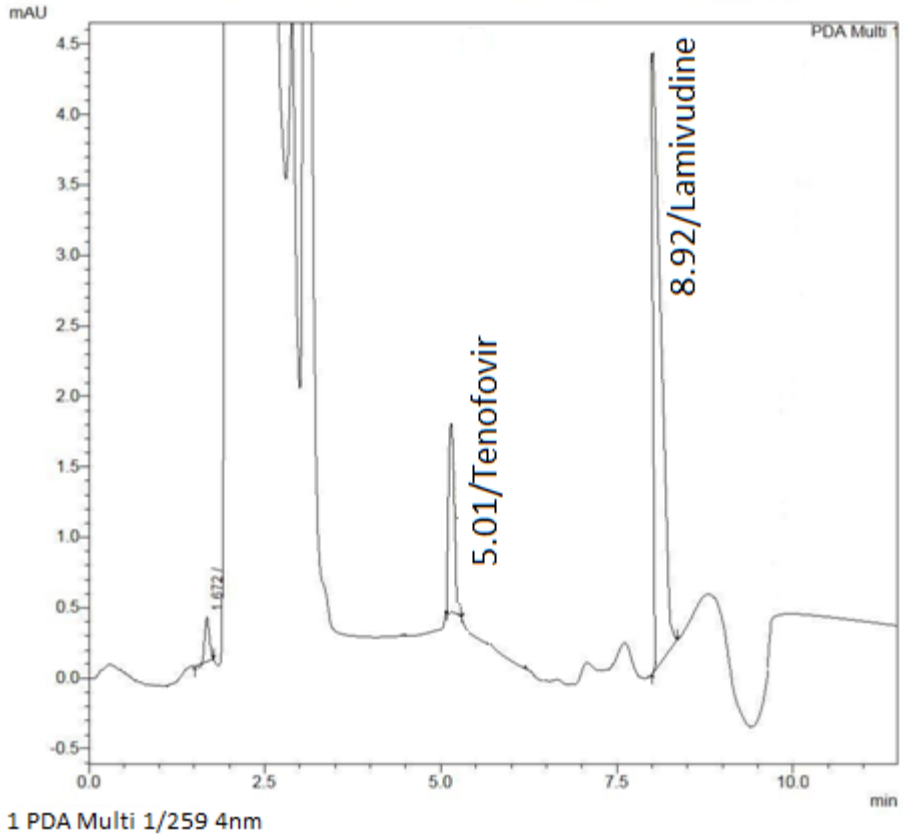

Fig: 10 Chromatogram of Tenofovir and Lamivudine in plasma 400ng/ml

==== Shimadzu LCsolution Analysis Report ====

C:\Documents and Settings\Administrator\Desktop\New Folder (2)\sample 024.lcd
Acquired by: KMCH College of pharmacy
Sample Name: plasma acn meth sample ph 3
Sample ID: Sample
Injection Volume: 20 ul
Data File Name: spiked plasma 800ng.lcd
Method File Name: sample.lcm
Batch File Name:
Report File Name: Default.lcr
Data Acquired: 2/21/2012 1:18:51 PM
Data Processed: 2/21/2012 2:57:50 PM

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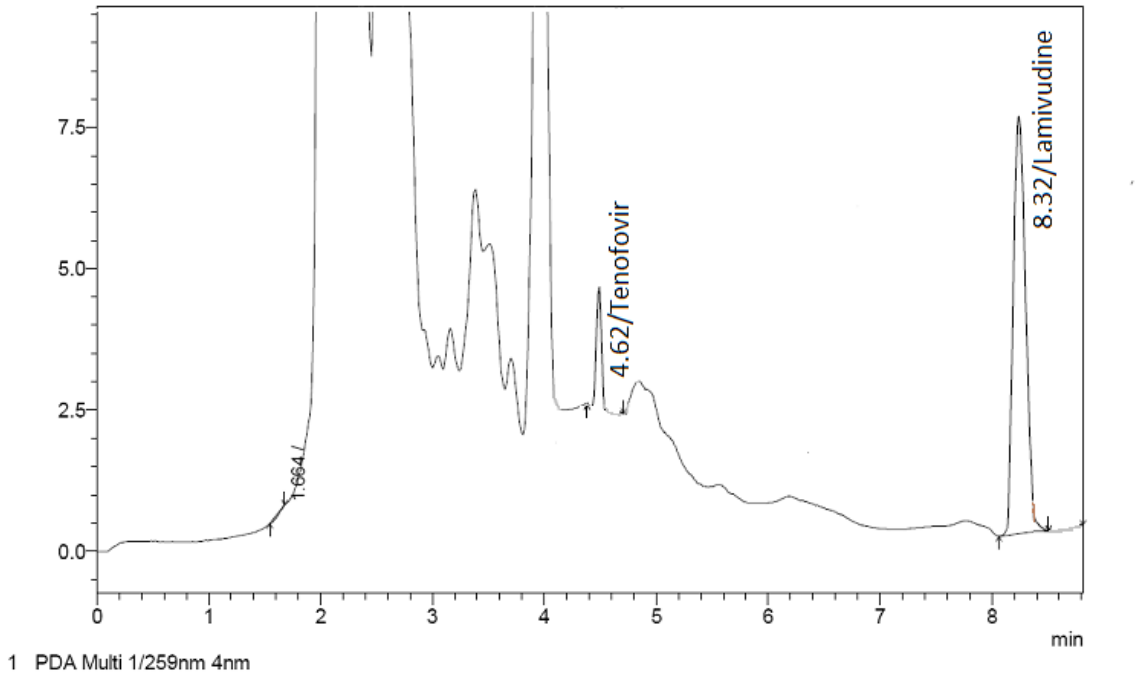

Fig: 10 Chromatogram of Tenofovir and Lamivudine in plasma 600ng/ml

==== Shimadzu LCsolution Analysis Report ====

C:\Documents and Settings\Administrator\Desktop\New Folder (2)\sample 027.lcd
Acquired by: KMCH College of pharmacy
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Sample ID: Sample
Injection Volume: 20 ul
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Method File Name: sample.lcm
Batch File Name:
Report File Name: Default.lcr
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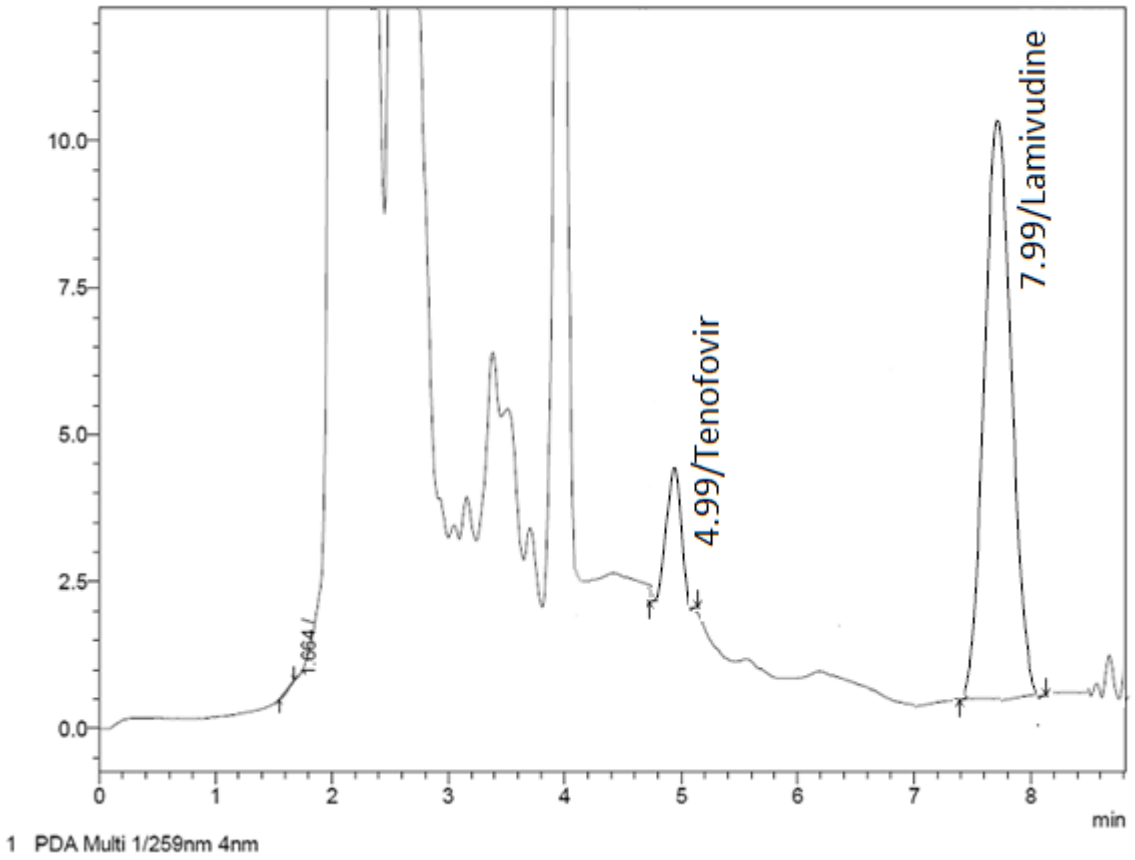

Fig: 10 Chromatogram of Tenofovir and Lamivudine in plasma 800ng/ml

==== Shimadzu LCsolution Analysis Report ====

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Acquired by: KMCH College of pharmacy
Sample Name: plasma acn meth sample ph 3
Sample ID: Sample
Injection Volume: 20 ul
Data File Name: spiked plasma 1000ng.lcd
Method File Name: sample.lcm
Batch File Name:
Report File Name: Default.lcr
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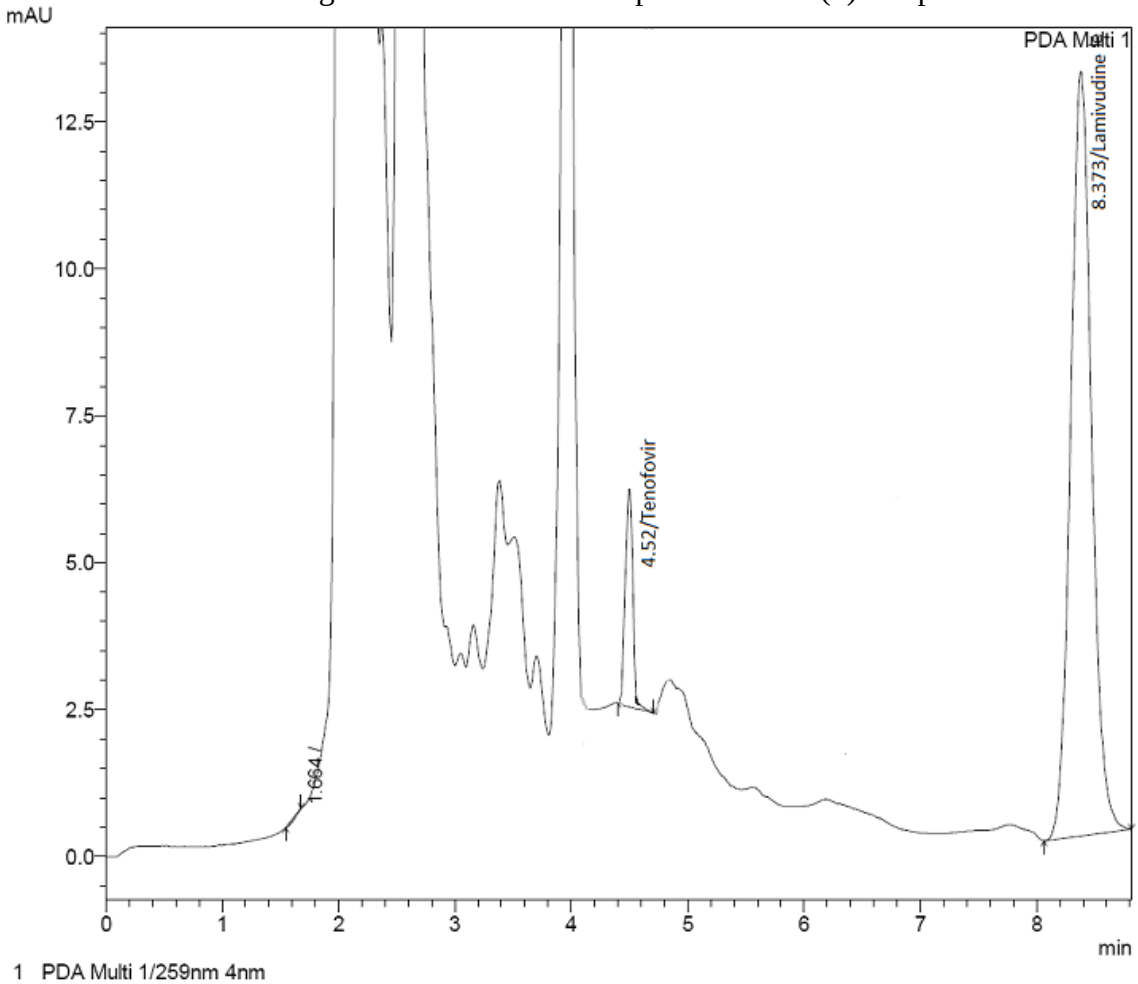
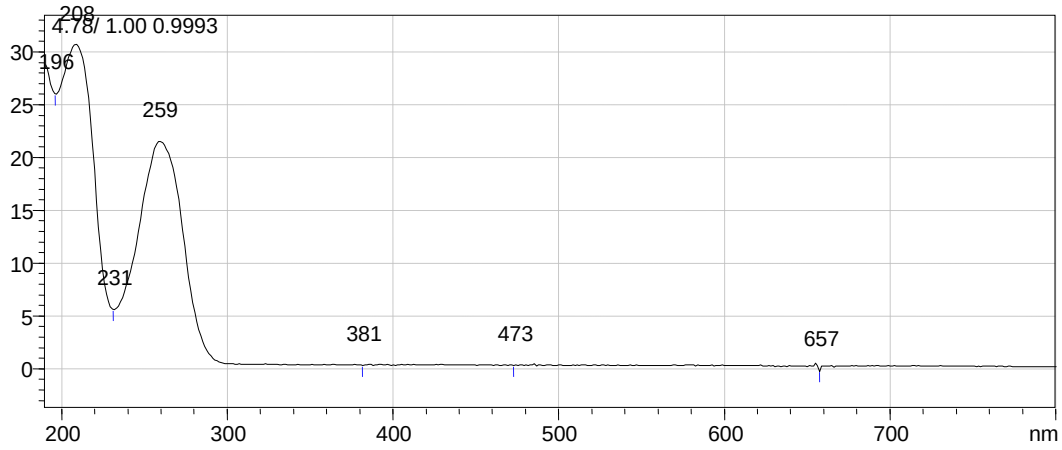
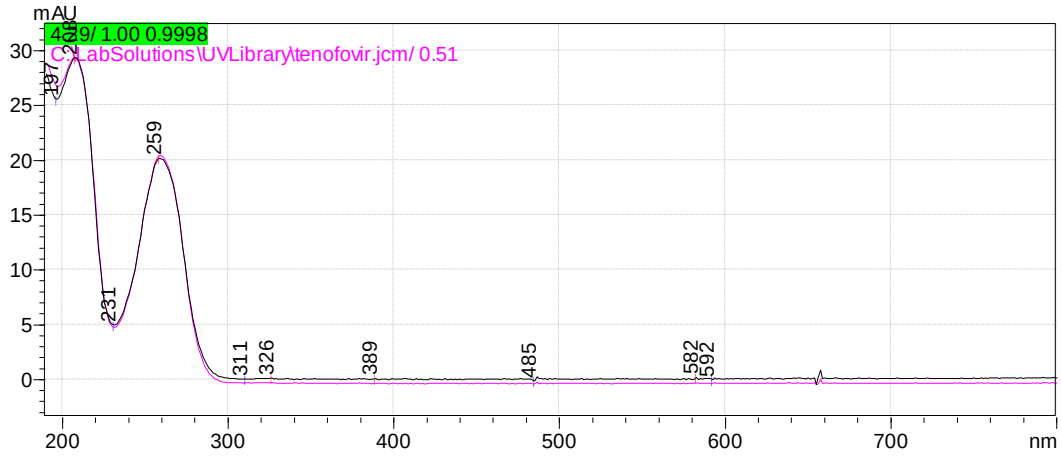


Fig: 10 Chromatogram of Tenofovir and Lamivudine in plasma 1000ng/ml

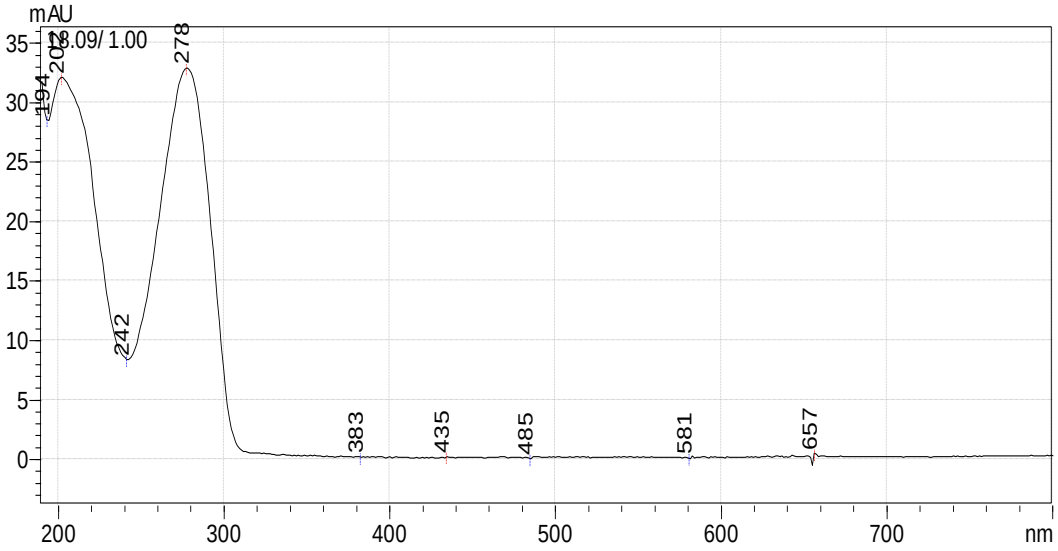
UV SPECTRUM



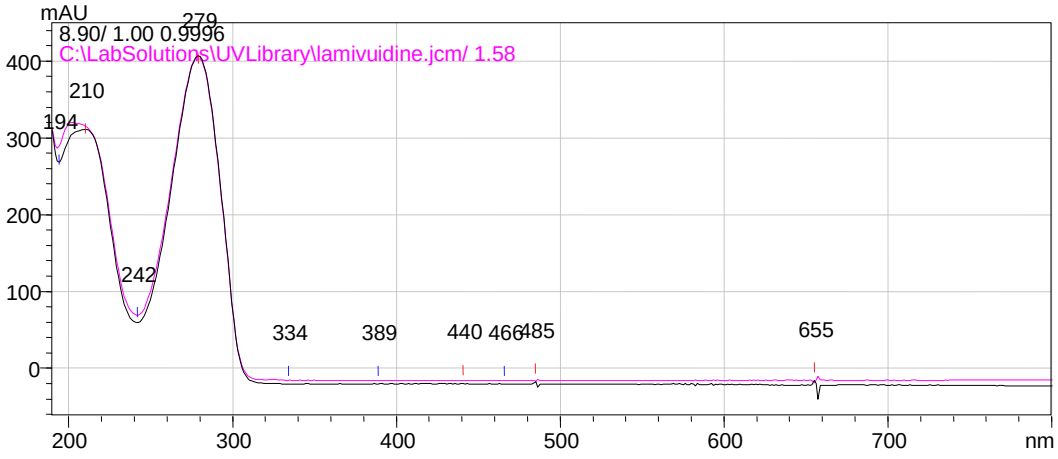
UV spectrum of standard Tenofovir



UV spectrum of plasma matches with Tenofovir spectrum



UV spectrum of standard Lamivudine



UV spectrum of plasma matches with Lamivudine

PEAK PURITY GRAPHS

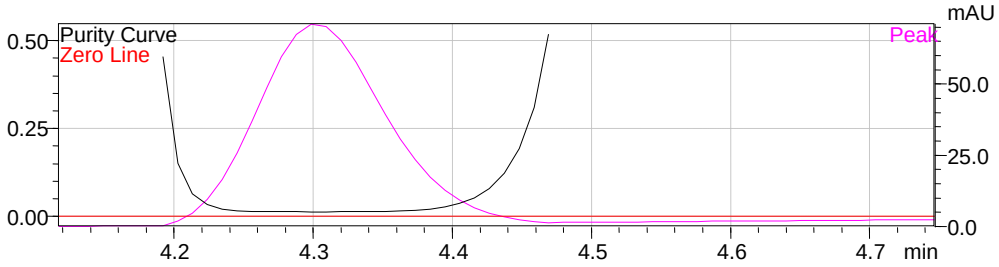


Fig: 16 Peak purity of standard Tenofovir

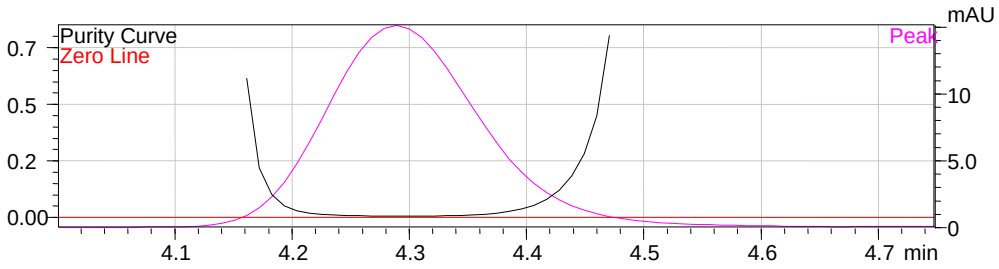
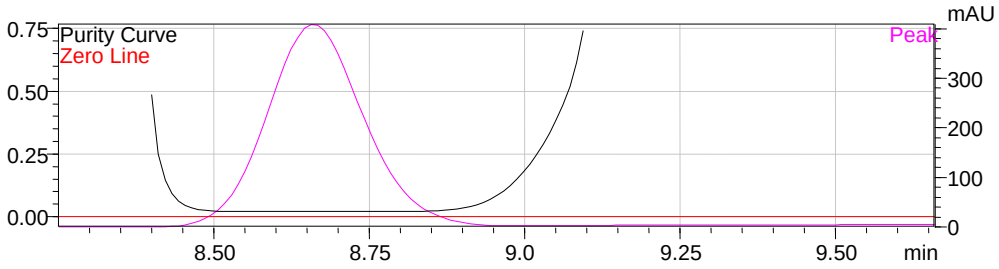
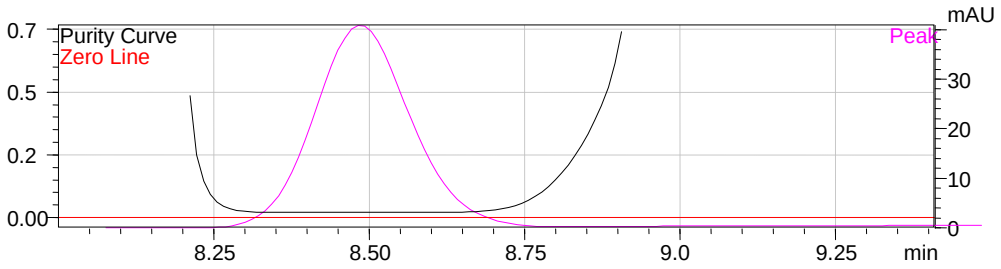


Fig: 17 Peak purity of Tenofovir in plasma

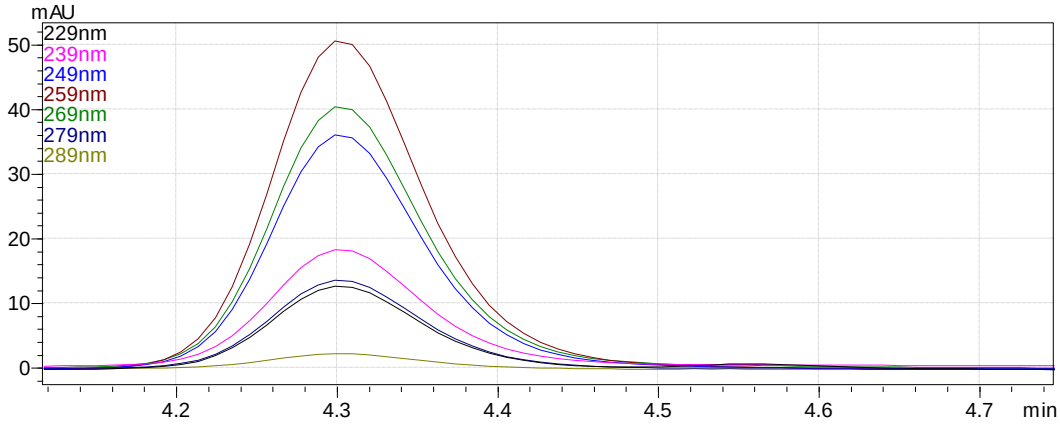


Peak purity of standard Lamivudine

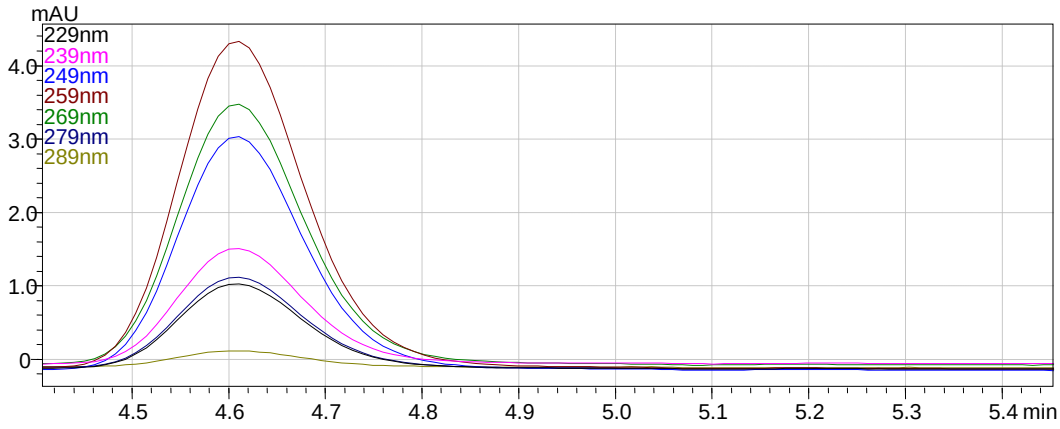


Peak purity of Lamivudine in plasma

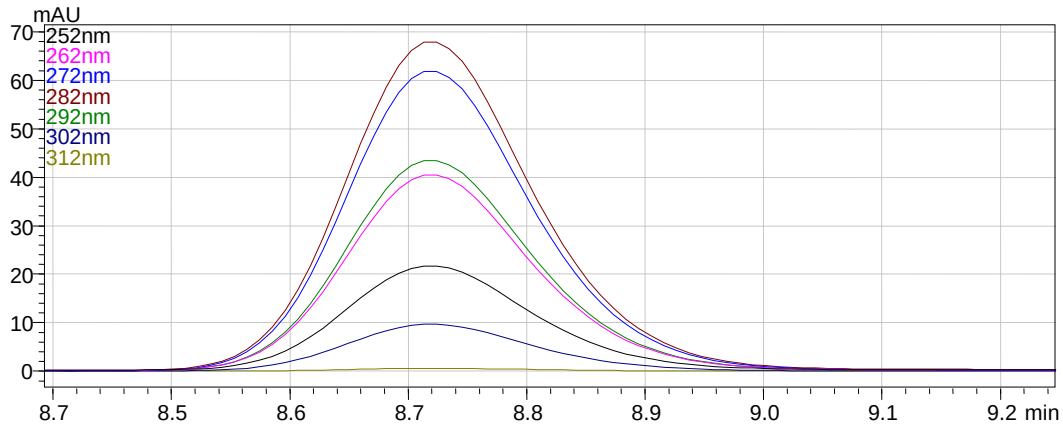
Peak profile



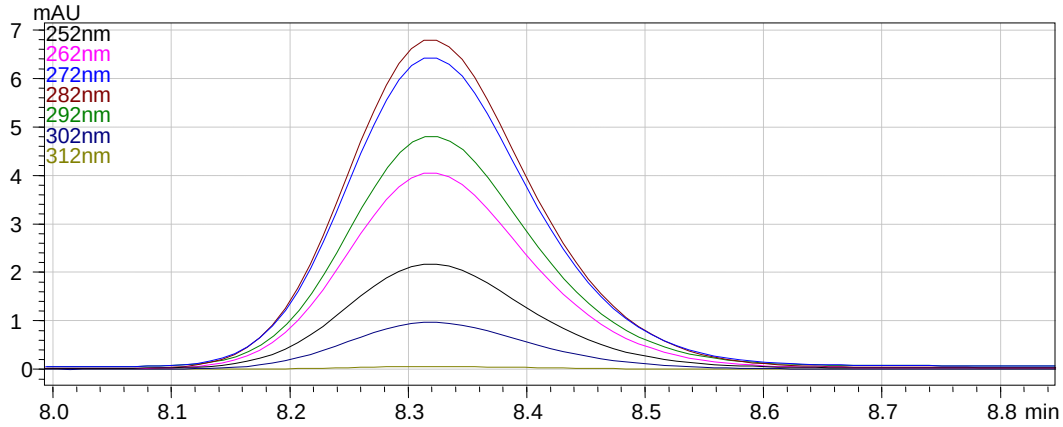
Peak profile of standard Tenofovir



Peak purity of Tenofovir in plasma



Peak purity of Lamivudine



Peak purity of Lamivudine in plasma

5. SUMMARY AND CONCLUSION

A bioanalytical method was developed for the estimation of Lamivudine and Tenofovir Disoproxil Fumarate by HPLC method. The method was validated for its transferability to other user or other laboratory. The HPLC method developed by using Heptane sulphonic acid with pH 3.0 adjusted with orthophosphoric acid and acetonitrile in meticulous ratio. The peaks obtained for the drugs of interest by the present method are well resolved from each other without any interference and from the plasma endogenous proteins. The peaks are symmetrical with acceptable tailing factor. The retention time of Lamivudine and Tenofovir Disoproxil Fumarate was shorter and proves the method is rapid.

The results of linearity, intraday and interday precision study and capability of the extraction method were within the limits of bioanalytical method development. The method was linear with a correlation coefficient of acceptable agreement, which is suitable for the estimation of Lamivudine and Tenofovir Disoproxil Fumarate in human plasma and other biological fluids.

The method demonstrated relative recoveries with acceptable relative standard deviation. The limit of quantification (LOQ) and limit of detection (LOD) for Lamivudine and Tenofovir Disoproxil Fumarate was found to be micrograms lesser than unity. Hence the developed method is sensitive for the estimation of Lamivudine and Tenofovir Disoproxil Fumarate in trace amounts.

Peak purity studies, with peak purity index values closer to unity reveals that the method developed was specific for the estimation of Lamivudine and Tenofovir Disoproxil Fumarate in blood and other biological fluids.

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